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ENZYMES

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ENZYMES

PROPERTIES, DISTRIBUTION, METHODS AND APPLICATIONS

BY

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PREFACE

It has been our endeavor to collect in as concise form as possible the available information in regard to enzymes and to indicate the original sources from which more detailed knowledge may be obtained. Anyone who attempts a study of enzymes cannot fail to be struck by the vast accumulation of literature on the subject, while at the same time he will be confused by the many apparently contradictory results that have been published. Over two thousand references have been consulted—most of them in the original—the others in abstracts which have appeared in various journals. To piece these irregular and loosely fitting fragments together into a mosaic upon which future studies may rest is the object of this book. Especial attention has been paid to the occurrence and preparation of enzymes, to the methods of measurement, and to the practical application of enzymatic activity—phases of the subject which are frequently overlooked in treatises on plant and animal physiology, as well as in those especially devoted to enzymes.

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S. A. W.
W. C. D.

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A. PROPERTIES OF ENZYMES

A. PROPERTIES OF ENZYMES

CHAPTER I

INTRODUCTORY

1. HISTORICAL DEVELOPMENT OF THE CONCEPTION OF ENZYMES. Prior to the 19th century, very little was known of the mechanisms of digestion, putrefaction and fermentation, although these terms had been in use almost from the time of Hippocrates. The word *digestion* was employed to designate any reaction in which organic matter was decomposed by organic juices. If the process was accompanied by the formation of foul smelling substances, it was defined as *putrefaction*. If gas was liberated, *fermentation* was said to be taking place. Fermentation was also differentiated into the spirituous, the acid and the putrefactive variety.

The first scientific work toward the elucidation of the phenomena of digestion was done early in the seventeenth century by Van Helmont, who demonstrated that acid took an active part in the digestion of food in the stomach. The next contribution was by Lavoisier[711a] two hundred years later, namely that alcoholic fermentation consisted of a chemical decomposition of sugar into alcohol and carbon dioxide. This process he believed to be theoretically reversible, and assumed that it was due to the presence of a "ferment." Lavoisier, however, was more interested in the reaction itself than in its cause.

A definite advance in the conception of enzymes began with the 19th century. In 1815 Kirchhoff[665] discovered that starch was dissolved by dilute acids when heat was applied and that it was finally transformed into sugar. The acid itself did not change in quantity and did not form any compounds with the starch or its derivatives. The qualitative and quantitative relationships in alcoholic fermentation were established in 1815 by Gay-Lussac. It was next reported that oxygen was liberated from H_2O_2 when treated with silver, gold, platinum or blood pigment. Of special interest were the observations made in regard to spongy platinum, which was found to be capable of causing a combination between hydrogen and oxygen. This and allied phenomena were termed *catalyses* by

Berzelius [131] who defined a catalyst as a substance which, by its mere presence, is capable of awakening the energies slumbering at given temperatures. Berzelius recognized the similarity between the fermentation of sugar and the decomposition of H_2O_2 and suggested that a correlation existed between fermentation and catalysis. He spoke of alcoholic and acid fermentation and putrefaction as catalytic life processes, or the results of the catalytic power of organic tissues. A "ferment" he looked upon as a catalyst which was capable of causing alcoholic and acid fermentation. The insoluble, nitrogen-containing sediment, formed from pressed grape juice by oxidation, was regarded as the yeast or ferment which was capable of bringing about the fermentation of grape sugar solutions. Liquids and infusions, which offered favorable media for the development of microörganisms, were thought to be producers of ferments, for at that time microbiology was an undiscovered science. Acetic and lactic acid fermentations were considered to be the second stage of decomposition of plant substances, putrefaction as the third.

In 1830, Dubrunfaut made an extract of malt which converted starch into sugar, and in 1833 Payen and Persoz[956] precipitated, from malt extract, by means of alcohol, a substance capable of digesting starch. This substance which they called *diastase* could be dried and preserved.

The discovery of *emulsin* in bitter almonds by Robiquet, *pepsin* in the stomach by Eberle and Schwann[1109] and *trypsin* in pancreatic juice by Corvisart, all catalytically active nitrogen-containing substances, seemed to confirm Berzelius' conception that yeast precipitate was also a ferment, or a purely catalytic substance. However, the idea of Schwann that yeast was a minute reproducing organism, at first opposed by Berzelius, was gradually gaining ground. In 1839 Liebig attempted to explain fermentation as a transmission of the agitation of the molecules, which leads to their breaking away from one another thus causing a chemical decomposition of the fermenting material. According to this idea alcoholic fermentation would be brought about by the decomposition of yeast; protein decomposition by pepsin would be due to the breaking up of the latter and so forth.

Pasteur by his brilliant contributions proved that the formation of alcohol and CO_2 from grape sugar was a result of the life activities of yeast cells. He defined fermentation as "life without oxygen," a phenomenon analagous to the respiration of organisms which can live without atmospheric oxygen. According to this theory, alcoholic and other fermentations are closely connected with the life of living organisms; these ferments being *"formed"* or *"organized"* in nature, in contradistinction to *unorganized ferments*, like diastase or emulsin, whose activities do not require the presence of living cells.

The indiscriminate use of the word "ferment" was bound to produce confusion so in 1867 Kühn[694] suggested the name *enzyme* (from the Greek εν ζύμη, in leaven) for all *unorganized* or *unformed* ferments. The mechanists (Berthelot, Hoppe-Seyler) regarded "ferments" as active agents within the living cells which carried on their catalytic processes inside of the cell; when these agents were secreted outside of the cell, they called them "enzymes." The vitalists, however[864], claimed that deep-lying differences existed between fermentations and enzymatic reactions, the former occurring only in immediate contact with the living cell, while enzymes acted when separated from the cell. The terms "bio-chemical catalyst"[363] and "bio-catalyst"[109] have also been suggested for enzymatic reactions.

The last link in the development of the conception of enzymes was the work of the Buchner and Hahn[206–213] on cell-free fermentation. By crushing the yeast cells with sand, and then pressing out the juice, they were able to obtain a substance free from cells which was capable of producing alcoholic fermentation. With the discovery of the enzymes of acetic and lactic acid bacteria, a series of "endoenzymes" was obtained. Although living protoplasm is the carrier and producer of the enzymes, it can often be destroyed without injuring the enzymes. The difference between ferment and enzyme then disappears. It is possible that, in addition to enzymes, other catalytic processes are active within the cell, as in catalysis by heavy metals and other surface catalysts such as charcoal.[1249]

Finally, the work of Harden and Young[519] on the co-enzyme of zymase (see p. 19), that of Sörensen[1157,1158] and Michaelis[821–836] on the influence of the hydrogen ion concentration upon enzymatic reactions, the work of Croft Hill[563] on the synthetic action of enzymes and of Willstätter and his associates[1273–1289] on the preparation of enzymes have greatly extended the knowledge of the physico-chemical processes involved in enzymatic reactions. Enzymology, therefore, can now be looked upon as a branch of biology, which closely touches upon chemistry, physical-chemistry, colloidal chemistry and mathematics.

2. Definition and General Properties of Enzymes. A catalyst is any stubstance which alters the rate of a reaction that other wise would be comparatively slow. Catalysts do not initiate reactions but merely change the rate of a reaction already in progress. They themselves are not used up and do not appear in the final products of the reaction. Catalysts may be either positive or negative, that is, they may increase or decrease the rate of a reaction (Ostwald). The former are the more important. Catalysts do not increase the amount of energy necessary to produce the reaction, but apparently diminish the inner resistance, acting similarly to lubricating oil in an engine. The action of finely divided platinum in stimulating the combination of hydrogen and oxygen to form water, the hydrolysis of cane sugar by acids, the acceleration produced by iron and manganese in oxidations by hydrogen peroxide and other peroxides are all examples of catalytic action.

An enzyme is a catalyst produced by living organisms and cells. It is seldom an ideal catalyst; while inorganic catalysts are definite chemical substances, the chemical structure of enzymes is still unknown. Each enzyme acts only upon substances of a definite structural and stereoisomeric configuration and we have learned to recognize and differentiate enzymes, not by their chemical composition, but by their effects upon these substances, or substrates. The activity of inorganic catalysts is augmented when the surface area is increased, therefore enzymes being colloids in a dispersed form are very active. Since enzymes have an extensive surface area, they exhibit the characteristic phenomena of adsorption. Most authors believe that enzymes contain nitrogen, phosphorus, sulfur, calcium, iron and sometimes manganese (in plant enzymes).[1275] These chemical analyses, which are still indefinite, have led some authorities to regard enzymes as proteins, and even nucleo-proteins. However, according to Euler and others,[404,1220] enzymes are not proteins in nature, but are in a colloidal combination with the proteins in the cell and their sensitiveness in natural extracts is due to this combination. Willstätter[1289d] conceives of the molecule of an enzyme as consisting of a colloidal carrier and of a purely chemically active group. The nature of the carrier is variable, since each enzyme can be separated from the chemically defined colloidal companion without loss of its specific activity. Nevertheless it is not yet possible to separate completely the chemically active groups of the enzyme molecule from the protective colloid without loss of activity.

The behavior of enzymes and inorganic catalysts is very similar. The reaction velocity depends in both cases upon the amount of active substance added. The quantity of enzyme necessary to accomplish a certain change, just as in the case of inorganic catalysts, is small in comparison with the amount of substrate upon which it acts. Invertase can catalyze at least 1,000,000 times its weight of cane sugar without losing its activity to any considerable extent.[933] The action of both inorganic catalysts and enzymes is influenced by outside factors. Enzymes are, however, much more sensitive to acids and alkalies, are only active within certain limited temperature ranges, are easily destroyed by metallic salts and are thermolabile. Their action is usually limited and does not go beyond a certain transformation of the substrate. They tend to bring about a certain equilibrium and do not complete the reaction in one direction; they may form compounds with the substrate or reaction products; they may be more or less completely destroyed during the reaction. Enzymes usually bring about more complicated reactions than do other catalysts. The action of an enzyme is specific, each enzyme acting only upon substances of definite structure and stereochemical configuration, as will be shown later. Although, as has been stated, enzymes are regarded as catalysts the digestion of protein by pepsin is sometimes considered as a chemical reaction and as not catalytic in nature.[897]

Enzymes and the substrate form unstable combinations which are considered to be the active molecules in the reaction.[109,894,1213] In dilute solutions the rate of change is proportional to the concentration of the enzyme but in more concentrated solutions the rate does not increase as rapidly as the concentration of the enzyme. Enzyme reactions proceed most rapidly at certain definite hydrogen ion concentrations. One reaction may lead to another transformation and sometimes the energy produced in the first reaction is utilized for the second. Various intermediary products may be formed. Some of the reactions caused by enzymes are exothermic and, therefore, a large amount of heat is produced; a gram-molecule of glucose transformed into alcohol and carbon dioxide liberates 71 calories, a gram-molecule of urea transformed into ammonium carbonate yields 8 calories. The heat set free is utilized by the cells for maintenance, as well as for the construction of new protoplasm. In hydrolytic processes, the end products have approximately the same heat value as the original substrate. Reduction phenomena are endothermic and absorb heat.

TABLE 1

Classification of enzymes

NAME OF ENZYME	SUBSTRATE	END-PRODUCTS FORMED
I. *Hydrolytic enzymes*		
1. *Esterases*	Esters	Acids + alcohols
Lipases	Fats	Higher fatty acids + glycerol
Butyrase or butyrinase	Lower esters	Lower fatty acids + alcohols
Lecithinase	Lecithin	Cholin + glycerophosphoric acid
Cholesterinase	Cholesterin esters	Cholesterin
Chlorophyllase	Chlorophyll	Alcohol + phytol + chlorophyllid
Phosphatase (zymophosphatase)	Organic phosphorus compounds (fructose-diphosphate)	R·OH (fructose) + phosphoric acid
Glycerophosphatase	Glycero-phosphoric acid	Glycerin + phosphoric acid
Phytase	Phytin	Inositol + phosphoric acid
Nucleotidase	Nucleotide	Nucleoside + phosphoric acid
Sulfatase	Ethereal sulfate	Phenol (or its homologues) + sulfuric acid
2. *Carbohydrases*		
(a) Polysaccharidases	Polysaccharides	Lower saccharides
Cellulase	Celluloses	Cellobiose
Cytases	Hemicelluloses	Dextrins and monosaccharides
Diastase (amylase)	Starch and dextrins	Dextrins and maltose
Inulase	Inulin	Fructose
Pectinase (pectase)	Pectic substances	Reducing sugars
Seminase	Mannogalactans	Mannose, galactose
Lichenase, etc.	Lichenin, etc.	Monosaccharides
(b) Trisaccharidases	Trisaccharides	Disaccharide + monosaccharide
Raffinase	Raffinose	Melibiose + fructose
Gentianase	Gentianose	Gentiobiose + glucose
Melezitase	Melezitose	Turanose + glucose

(c) Disaccharidases	Disaccharides	Monosaccharides
Invertase (α-fructosidase)	Sucrose	Glucose + fructose
Maltase (α-glucosidase)	Maltose	Glucose
Trehalase (α-glucosidase)	Trehalose	Glucose
Lactase (β-galactosidase)	Lactose	Glucose + galactose
Melibiase (β-galactosidase)	Melibiose	Glucose + galactose
Cellobiase (β-glucosidase)	Cellobiose	Glucose
Gentiobiase (β-glucosidase)	Gentiobiose	Glucose
(d) Glucosidases	Glucosides	Glucose + other products
α-glucosidase (other than maltase and trehalase)	α-glucosides	Glucose + alcohol or phenol residue
β-glucosidase (emulsin, phenol-β-glucosidase)	β-glucosides	Glucose + alcohol or phenol residue
Amygdalase (oxynitril-β-glucosidases)	Amygdalin (nitril-glucosides)	Glucose + benzaldehyde + hydrocyanic acid
Myrosinase	Sinigrin (myrosin)	Glucose + potassium-hydrogen-sulfate + allyl isothiocyanate
Nucleosidase	Nucleoside	Pentose + purine bases
Tannase	Tannin (digallic acid)	Gallic acid

TABLE 1—*Continued*

NAME OF ENZYME	SUBSTRATE	END-PRODUCTS FORMED
I. *Hydrolytic enzymes—Con.*		
3. *Enzymes acting upon Proteins*		
(a) Coagulating enzymes		
Thrombin	Fibrinogen	Fibrin
Rennet (lab)	Casein	Paracasein
(b) Proteases		
Pepsin	Proteins	Albumoses, peptones
Trypsin	Proteins, albumoses, peptones, peptides	Peptides, amino acids
Erepsin	Albumoses, peptones, peptides	Amino acids
Plant and microbial protease	Proteins, peptones, peptides	Peptones, peptides, amino acids
(c) Nuclease	Nucleic acids	Sugar, purine bases and phosphoric acid
(d) Amidases		
Desaminases	Amino acids	Hydroxy-acids + ammonia
Desamidases	Acid amides	Acids + ammonia
Arginase	Arginine	Urea + ornithin
Urease	Urea	$(NH_4)_2CO_3$
Purine base deamidases		
Guanase	Guanine	Xanthine + NH_3
Adenase	Adenine	Hypoxanthine + NH_3
Histozyme	Hippuric acid and its homologues	Glycocoll + benzoic acid (or their homologues)
Nitrilases	Oxynitriles	Aldehyde + HCN

II. *Oxidases, oxido-reductases and zymases*		
1. *Oxidases*		
Phenolase	Phenols	Quinones
Tyrosinase	Tyrosine	Melanins
2. *Peroxidases*		
Peroxidase	Peroxides	Reduction products of peroxides and active oxygen
3. *Oxido-reductases*		
Schardinger enzyme	Methylene blue + aldehyde	Leuco compounds
Aldehydases	Aldehydes	Acids and alcohols
4. *Purine oxidases*		
Xanthoxidase	Xanthine	Uric acid
Uricase	Uric acid	Allantoin
5. *Zymases*		
Zymase	Glucose, fructose, mannose, galactose	Alcohol + carbon dioxide
Glycolase	Sugars	Lactic acid
Glyoxalase	Methyl-glyoxal	Lactic acid
Carboxylase	Pyruvic acid	Acetaldehyde + carbon dioxide
Fumarase	Fumaric acid	Malic acid
III. *Catalase*	Hydrogen peroxide	Water + molecular oxygen

The chemical processes carried out by enzymes can be divided into hydrolytic reactions in which changes occur with the addition of water; oxidation phenomena in which oxygen is added or hydrogen is removed; reduction processes in which hydrogen is added or oxygen is removed, this and the preceding process often going hand in hand; so-called fermentations, which consist in the decomposition of complex organic substances into simpler compounds, without the participation of atmospheric oxygen, these processes liberating energy; and finally synthetic processes. There is some basis for the assumption that enzymes, unlike inorganic catalysts, may even initiate a reaction.

3. Terminology and Classification. There are a large number of enzymes described in the literature (Table I). The existence of some of these has not as yet been definitely established. Only the better known ones will be considered in this book. Since our knowledge of the true nature of enzymes is very vague, the chemical changes they affect must be used as a basis for classification. There are at least four systems of nomenclature for enzymes: 1, by combining the termination *"ase"* with the name of the substrate upon which they act, e.g., amylase, urease, and protease;[352] 2, by combining this ending with the name of one of the end products formed in the reaction, as alcoholase and glucase; 3, according to the nature of the reaction taking place, as oxidase, reductase and invertase, and 4, by the use of terms which have no connection with the process involved, e.g. zymase, emulsin, pepsin and trypsin. The latter often designate specific preparations, which may contain a number of enzymes. It has been suggested that synthesizing enzymes should be named by the addition of the ending *"ese"* to the substance formed. However, since there is no evidence that such enzymes exist apart from those producing hydrolysis, this terminology is probably unnecessary.[109]

An enzyme which digests starch is spoken of as amylolytic or amyloclastic, those attacking protein as proteolytic or proteoclastic, etc. Often a cell does not secrete the complete enzyme but a mother substance, which is transformed into an active enzyme outside of the cell. This is termed a *proenzyme or zymogen.* The activation of these mother substances is accomplished either by means of chemicals, as in the action of hydrochloric acid on pepsin, or by special substances called *kinases.* Certain substances resistant to heat whose presence may be necessary for the action of specific enzymes are *co-enzymes* or *activators.* The *anti-enzymes* are substances which inhibit the action of the enzymes.

CHAPTER II

Enzymes in Biological Processes

1. Rôle of Enzymes. Enzymes are of biological origin and are of great importance in the various activities of living matter. Biological processes must be differentiated into those performed by enzymes and those by living protoplasm. The enzymes enable the cell to carry on the various hydrolytic and synthetic processes. Within the cell they transform organic and inorganic substances for the production of energy and also play a part in various intracellular syntheses. Outside of the cell they digest nutrients so as to facilitate their entrance into the cell. The latter process is especially true of microörganisms, with which the formation of exoenzymes is easily demonstrated. In the case of bacteria, protozoa and other microscopic organisms, as well as in various animal organs, the nutrients are either first transformed by the extracellular enzymes or are taken up by the cell sap and then after ingestion by the cell are digested by the intracellular enzymes.

In the living cell, the activities of the enzymes produced by the cell and the processes carried on by the protoplasm of the cell itself may go hand in hand. In the decomposition of proteins by bacteria, for example, the phenomena of hydrolysis into peptones, amino acids and ammonia, which are purely enzymatic, are usually accompanied by purely biochemical processes (which themselves may be accelerated by certain specific enzymes), such as reduction, synthesis, utilization of the carbon part of the molecule for energy, etc.

The rôle of the digestive enzymes has been studied most extensively, but much of our information is still indefinite; even less is known of the tissue enzymes, while the part played by the oxidative and reducing enzymes in biological processes is still the subject of speculation. The function of the digestive enzymes in the intestinal canal, for example, consists in converting the nutrients into substances which can be absorbed. The hydrolysis of the fats begins in the stomach, but is largely completed in the intestine, by means of the lipase from the pancreas; the fatty acids and glycerin are then absorbed and resynthesized. Starch is first acted upon by the salivary amylase, the process continued in the stomach until stopped by the high acidity and is completed in the intestine by the pancreatic and intestinal amylase and maltase. The final product—dextrose—is then absorbed. Cane sugar and lactose are also hydrolyzed in the intestine by their corresponding enzymes. The proteins are digested by a series of enzymes, first by the pepsin of the stomach, then by the trypsin of the pancreas and the erepsin of the intestine. The hydrolytic products, largely amino acids, pass through the intestinal wall into the body fluids, where begin the processes of metabolism with which the enzymes of the blood, cells and organs are concerned. Thrombase, proteases, carbohydrases, esterases and the so called "protective ferments" are found in the blood. Among the tissue enzymes, the most important are those concerned in processes of oxidation which lead to the liberation of energy and those of synthesis; hydrolysis may often take place in the body, especially in connection with the utilization of the reserve substances. Since plants obtain their nutrients in the form of simple inorganic substances, the plant enzymes are largely synthetic in nature but the decomposition of fats, carbohydrates and proteins, which occurs in the intermediary metabolism of plants, is accomplished similarly to that in animals. The formation of reserve substances, especially starches, and to a lesser extent hemicelluloses, fats, glucosides and proteins, plays an important rôle in plant life, especially in the formation of seed.

2. Secretion of Enzymes. In the case of animals the *free* (*exo*) *enzymes* are formed and secreted by special organs (glands); some glands such as the salivary may secrete only one type of enzyme; others, like the pancreas, may secrete several enzymes. The course of the secretion of an enzyme is, in the case of secretion of pepsin by the stomach, a monomolecular reaction.[404] Various predacious insects secrete in the saliva an enzyme which digests the proteins before they are taken into the system.[632, 688] In the case of micro-organisms, the enzymes are secreted into the culture medium in which they are growing. Enzyme secretion also takes place in plants, in the form of juices of certain fruits, such as pineapple and papaw. The *intra-cellular or endo-enzymes* may be obtained only after the cell is ruptured as in the preparation of zymase by the Buchner pressure method. The endo-enzymes may also be liberated during autolysis after the cell dies. This process has been utilized for the preparation of invertase.[714] Other enzymes are

not readily separated from the cell, as in the case of those of the lactic and acetic acid bacteria. Only a rapid destruction of the cell with acetone permits the conservation of certain endo-enzymes. Some cannot be extracted from living or even dead cells and may even be destroyed during the process of killing the cells. It is impossible, therefore, to conclude from the absence of certain enzymes in a bacterial culture or in the pressed juice of microörganisms or cells that a particular enzyme is not produced; the broken down cells themselves should be tested.[8]

3. Influence of Composition of Medium on Formation of Enzymes. The amount of enzyme produced by certain cells depends not so much upon the nature of the cells as upon the substrate in which they are grown.[149a,158,159,389–414,432,436,497a,983a] This difference in enzyme formation, under different conditions, is rather quantitative than qualitative in nature. There are comparatively few instances on record in which the formation of an entirely new enzyme is due to the presence of a specific substance in the medium or to specific conditions. This adaptation may not represent the formation of a new enzyme but merely a change in the constitution or configuration of a component of the enzyme complex.[394] It is not necessary to assume the existence of a specific enzyme for each separate process for it is possible that the same enzyme can catalyze various reactions depending upon the composition of the medium in which they occur. Since this medium can vary within the cell, various conditions may be produced which are optimal for various enzymatic processes.[603]

The amounts of carbohydrate splitting enzymes formed by fungi may be materially increased by cultivating the organisms in media containing the corresponding carbohydrate; this is especially true of amylase, inulase and lactase, which are ordinarily formed in small quantities.[339] The influence of the composition of the medium upon diastase formation by *Asp. niger* is illustrated in Table II. By growing *Asp. oryzae* in the corresponding substrates the formation of the following enzymes is stimulated: invertase,[699,1261] maltase, diastase,[339,404,650] inulase,[339,1313] and protease.[224,324,1237] The nitrogen source has an important influence upon the formation of diastase by *Asp. oryzae*[1072a] and *Pen. camemberti*,[1025] and the formation of emulsin by *Asp. niger*.[555] Zinc also influences diastase formation by *Asp. niger*.[612a] Oxygen increases the production of zymase by certain fungi.[635] A green Penicillium forms invertase only when grown on calcium lactate; grown on starch, it produces diastase; grown in milk, it yields a proteolytic enzyme.[352] *Asp. niger* and certain *Penicillia* grown on synthetic media, i.e., Czapek's solution, do not form the enzyme tannase but if, in addition to sucrose, tannic or gallic acid is present in the medium, tannase is produced by the fungi.[669] The greater the amount of tannic acid in the medium, the greater is the quantity of tannase formed. The production of nuclease by fungi which was at first thought to be dependent on the presence of nucleic acid in the substrate has now been found to be an independent phenomenon.[339,598]

TABLE II

The influence of the composition of the medium upon diastase production by Asp. niger[404]

MEDIUM IN WHICH THE CULTURE WAS GROWN	REDUCING SUGAR PRODUCED BY 1 GRAM OF DRY MYCELIUM, IN 2 HOURS AT 40°C. AND pH 6.5 TO 7.0
	mgm.
10 per cent cane sugar	14.9
10 per cent cane sugar + 1 per cent peptone	41.0
2 per cent cane sugar	17.6
2 per cent cane sugar + 1 per cent peptone	43.0
2 per cent starch	91.0
2 per cent starch + 1 per cent peptone	146.0

Definite chemical groups must be present in the medium to enable microörganisms to produce certain enzymes. Leucine is essential for the formation of urease by *B. proteus*, but not for catalase. The formation of the latter is greatly stimulated by lactic acid. Closely related substances may take the place of the specific substrates in the medium for the production of some enzymes.[604–606] The reaction of the medium also influences the formation of urease by *B. proteus*, the maximal enzyme formation taking place at pH 7.0.[1185] The presence of starch in the medium increases the formation of diastase by various microörganisms,[203a] while sugar inhibits the formation of the enzyme; the degree of inhibition depends on the nature and concentration of the sugar and on the type of organism.[650,1306] The presence of substances which diminish the utilization of a particular nutrient in the substrate brings about a diminution in the production of the corresponding enzyme. This is especially true in the formation of a gelatin liquefying enzyme by bacteria, which is diminished by the presence of sugar in the gelatin media.[54a,658,666a]

Yeast which cannot invert cane sugar is able to do so when grown in media containing equal parts of cane sugar and glucose;[350a] similar observations have been made with galactase and galactose.[324a,520,666b,1148a] The source of nitrogen influences the amount of invertase produced by yeasts.[436] The enzymes produced by *Monilia sitophila* may be divided into three groups: (a) those formed in at least small amounts when the organism is grown on any medium (amylase, invertase and tyrosinase); (b) those produced only when certain substances closely allied to their substrates are present; (c) those which are formed only when the particular substrate upon which they act is present in the culture medium (trypsin, rennet, trehalase).[1261]

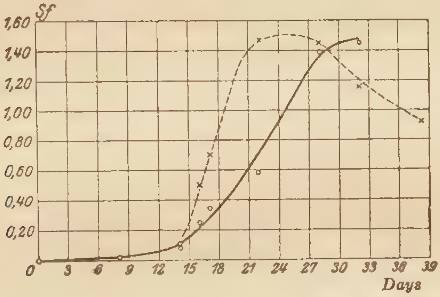

FIG. 1. COURSE OF FORMATION OF DIASTASE IN PLANTS (*Phaseolus vulgaris*)
ooo - cotyledons, xxx - leaves[1147]

Invertase formation by yeasts also depends on the temperature and reaction of the medium.[403,407] While the optimal temperature for the growth of yeasts is 23.5°C., the optimum for invertase formation is 27.5°C. The optimal reaction for the formation of invertase is pH 5.6. Similar phenomena have been noted in the higher plants.[200] The abundance of diastase in leaves in the morning and its diminution during the day are apparently results of temperature changes. Resting seeds either contain no enzymes or only very small amounts, but as soon as germination occurs, the necessary diastatic, proteolytic, lipolytic, cellulose-splitting and other enzymes are formed by the embryo (Fig. 1). In germinating plants the amount of amylase produced is adapted to the nature of the nutrient.[1146,1147]

This adaptation phenomenon was observed in insects by Claude

Bernard;[125,1173a] he found that larvae of *Musca lucilia* contained glycogen, but no enzyme which could hydrolyze it. However, as soon as the larvae changed into chrysalids a diastatic enzyme was formed and the glycogen was utilized.

The quantitative regulation of enzyme formation in animals is strikingly illustrated by the work of Pavlov on the effect of different nutrients upon the secretion of pepsin by the stomach, and of trypsin, diastase and lipase by the pancreas.[954,955] The feeding of milk causes a greater secretion of lipase than a diet of bread or meat which contains the same amount of nitrogen, while a bread meal produces a larger quantity of diastase. The secretion of protease is similar after each of these three foods. On the other hand, the enzyme activities of the pancreas and stomach of horses are unchanged whether vegetable or animal protein is fed. The enzymes of meat eating plants can digest all types of protein equally well. If meat or eggs are fed to seven day old infants, the gastric juice acquires the ability to digest these foods but this adaptation apparently depends upon an increase in gastric acidity for if the latter does not occur, the eggs are not digested.[538]

4. Proenzymes or Zymogens. Various enzymes are secreted in an inactive form (zymogens) and are later transformed by an activator into active enzymes. This is true of pepsin, trypsin and thrombin, which are secreted as pepsinogen, trypsinogen, and thrombogen. These and other zymogens have actually been isolated. Acids, alkalies, salts or special kinases are the activating agents. The kinases are the most complicated of the activators. In some instances, activation involves the formation of complex organic salts. The activating effect of the salts of heavy metals on oxidases may be due to the fact that these salts are carriers of oxygen.

The transformation of an inactive zymogen into an active enyzme is an irreversible reaction.[109] The trypsin of the pancreatic juice is secreted as a zymogen into the duodenum. Here it meets the enterokinase and is converted into active trypsin. The activation by enterokinase is an autocatalytic process, starting slowly and increasing in rate until the reaction is practically at an end. Calcium can also activate trypsinogen. Except for the fact that it is not proteolytic, trypsinogen has properties similar to those of the active enzyme.[109]

Esterases are often present in an inactive condition, as in the case of the pancreatic esterase. The zymogen is activated by bile salts as well as by synthetic sodium glycocholate and taurocholate.[778] This action of the bile salts is specific for the pancreatic esterase but does not affect stomach or intestinal lipase. The optimal concentration of the bile salts is 0.33 per cent, except in the hydrolysis of triolein, for which no optimal point has been observed.[1194]

A zymogen may also be regarded not as a preliminary stage of an enzyme but as a mixture of the latter with a paralyzing substance. According to this view, activation consists in the destruction of the paralyzer.[923]

5. Co-ENZYMES. Co-enzymes or accelerators are substances which increase the activity of enzymes. The addition of a minute quantity of manganese salts causes a great increase in the oxidizing power of laccase.[128c] Similar phenomena are illustrated by the influence of calcium salts on the activity of pectase, of asparagin on diastase, and of KCl, NaCl, $CaCl_2$ and KNO_3 on the diastase of dialyzed pancreatic juice.

When an extract of liver is dialyzed the lipase is divided into two fractions, each of which is separately inactive.[778] The non-dialyzable portion is destroyed by boiling while the dialyzable fraction which is the co-enzyme is thermo-stable. Sodium cholate and glycocholate possess the properties of co-enzymes. The action of the bile salts is believed to be due to their ability to produce a larger active surface of the colloidal enzymes by preventing aggregation of the molecules. Similar observations have been made on zymase; if yeast juice is filtered through a Martin gelatin filter (5 per cent gelatin on paper), the colloids left behind are inactive, but become active again when mixed with some of the filtrate.[519] The latter is inactive by itself. The filterable co-enzyme is dialyzable and thermo-stable and really consists of two co-enzymes, one of which is a phosphate. The nature of the other substance is still unknown although it may possibly contain an anti-protease. The reaction takes place as follows:

$$2C_6H_{12}O_6 + 2\ PO_4HR_2 = 2CO_2 + 2C_2H_5OH + \underset{\text{hexose-diphosphate}}{C_6H_{10}O_4(PO_4R_2)_2}$$

Alcoholic fermentation depends upon the concentration of sugar, phosphate and co-enzymes as well as upon the hydrogen ion concentration.[410]

6. ANTI-ENZYMES. Anti-enzymes are substances which have a marked inhibitory action on certain enzymes. Most of these agents

are probably not antibodies in an immunological sense. It has been maintained, however, that anti-enzymes are antibodies formed either as a result of the normal presence in the body, or of the artificial injection into the body of enzymes which act as antigens. The formation of anti-enzymes does not depend upon the presence of co-enzymes. They are regarded by Euler[404] as "enzyme-antibodies." Bayliss,[109] however, doubts whether anti-enzymes can be produced in response to the injection of enzymes, although he admits the existence of substances having a markedly inhibiting action. An anti-enzyme against emulsin was the first one prepared.[562] Anti-enzymes have since been obtained against lipase, invertase, diastase, rennet, pepsin, trypsin[18,124] and leucoprotease.[618] Of special interest are the antitrypsin of blood serum, antiurease of blood serum and urine, antirennet and antithrombin. It is still uncertain whether these artificially prepared anti-enzymes are the same as those which occur naturally. Anti-enzymes usually do not resist higher temperatures than enzymes although castor oil antilipase is quite resistant and is only slowly destroyed at 80°C.[126] Most of the anti-enzymes seem to combine with the enzymes and thus inactivate them. The reaction may be merely one of adsorption. The question of paralyzers will be considered in detail elsewhere (see pp. 52–57).

7. Specificity of Enzyme Action. The specific nature of enzymes as well as of inorganic catalysts is expressed in at least four ways: (a) The absolute and relative specificity of enzymes for substrates of different composition forms the present basis for the classification of enzymes. (b) Stereochemical specificity, i.e., enzymes may hydrolyze substances of a certain stereoisomeric configuration but not others of a slightly different configuration as in the action of proteolytic enzymes upon artificial peptides. (c) Different enzymes may produce different products from the same substrate, as illustrated in the action of invertase and emulsin upon raffinose:

$$\text{Raffinose} + H_2O + \text{invertase} \rightarrow \text{melibiose} + \text{fructose}$$
$$\text{Raffinose} + H_2O + \text{emulsin} \rightarrow \text{cane sugar} + \text{galactose}$$

(d) The same enzyme obtained from different sources may be affected differently by the reaction products, as for example, the invertase of yeast and that of *Asp. oryzae.*

a. Action of individual enzymes upon substrates of different composition. The classification and separation of enzymes rest upon their specificity, that is their ability to digest certain substrates and not others. The statement that a certain enzyme has no effect upon a substance indicates that negative results have been obtained in a test continued for a definite length of time, under certain conditions, and that the end-products were analyzed by certain methods, i.e., the velocity of the reaction, in the presence of the enzyme, is no different from the velocity without the enzyme. It is always possible that if the conditions of the experiment had been different or the methods more accurate, positive results might have been obtained.

Some enzymes are very limited in their effects, acting only upon a definite chemical compound, while other enzymes attack a large group of substances, bringing about a series of reactions. Invertase and catalase are examples of the former class. These two enzymes, which produce clearly specified reactions, have been used most extensively in the study of the kinetics of enzyme action.[450] Proteases, diastases and emulsin are examples of the class which acts upon a large group of substances. Attempts have been made to ascribe the various reactions of the members of this class to a series of separate enzymes rather than to one complex enzyme. This has led to the assumption of the existence of a large number of individual enzymes, which should more properly be considered merely as varieties of the same enzyme; for example, the enzymes gelatinase, casease, etc., are probably varieties of protease rather than distinctly different enzymes. However, the statement is frequently made that in the hydrolysis of proteins a number of enzymes are concerned which act upon specific substrates under a specific set of conditions and that trypsin for example consists of a true proteolytic enzyme, a peptolytic enzyme and different peptases for each polypeptide combination.[2–7] This plurality of enzymes has been suggested, not only for polypeptide-splitting enzymes, but also for others. Emulsin, for example, is often regarded as a mixture of at least three enzymes, two of which hydrolyze glucosides and a third (oxynitrilase) which converts the oil of bitter almonds into benzaldehyde and hydrocyanic acid. Emulsin also hydrolyzes amygdalin into glucose, benzaldehyde and HCN. Some investigators believe that emulsin is a mixture of five enzymes. Diastase is said to consist of several enzymes which progressively hydrolyze starch step by step into sugar.

Not only do different enzymes vary in the chemical changes they accelerate, but the same enzymes, of different origin, may react differently. Proteases of plants, for example, are distinctly different in their action from the proteases of microörganisms. This is also true of lipase and of emulsin. The diastase of *Asp. oryzae* will not only act under more acid conditions than will pancreatic diastase, but the very nature of the phenomenon is different. The former is more strongly starch liquefying while the latter, and this is also true of malt diastase, is more active in its sugar producing properties. Maltase of plants will hydrolyze α-methyl glucoside, but animal maltase will not.

Enzymes usually occur in mixtures, and only in isolated instances are individual enzymes found. In some cases, individual enzymes have been separated from these mixtures by precipitation, by autolysis, by the destruction of the other enzymes, and by selective extraction. Since the various individual enzymes in a mixture may be active under different conditions of temperature and reaction and since they also may be differently affected by activators they may be studied separately.

b. Action of enzymes upon stereoisomeric compounds. The specific action of enzymes is not only limited to the fact that proteases do not digest fats and carbohydrates, while lipases do not attack proteins and carbohydrates, but there are also certain limits to their activity within each group. Not only the qualitative chemical composition of the substrate but also the configuration of its molecules plays an important part. Apparently the stereochemical configuration of the enzyme may determine its ability to adapt itself to the configuration of the substrate molecule, a relationship which has been compared to that between a lock and key.[441–443] Of the sixteen possible stereo-isomers of hexose, of which twelve have been isolated or synthesized, only four can be attacked by the alcohol producing enzyme, zymase, namely d-glucose, d-mannose, d-levulose, and d-galactose. Of the stereoisomeric α- and β-methyl-glucosides, the former is readily and completely hydrolyzed by maltase, but never by emulsin, while the β-compound is completely hydrolyzed by emulsin but never acted upon by maltase. Chemical changes in the substrate, produced by the substitution or addition of carbon and other atoms affect the ability of an enzyme to digest this substrate. For example, if the structure of the β-glucoside is altered, the amount of hydrolysis produced by emulsin is changed. The glucosides of phenols and phenol derivatives are hydrolyzed more readily than the derivatives of aliphatic alcohols and their acids.

Synthetic peptides have been used to demonstrate the specificity of proteolytic enzymes. Many, but not all, of the synthetic peptides are digested by trypsin. When a racemic peptide is digested, the cleavage is an asymmetric one, and the amino acid split off is always the same as that found among the end products of a protein digested by trypsin.[441a] Fungus enzymes can act upon both isomers of amino acids, although the rates of the reactions are different.[990a]

Another interesting illustration of enzyme specificity in respect to configuration is the action of lipases obtained from different sources.[1289a] Lipase from the pancreas hydrolyzes glycerides ten to twenty times as rapidly as that from the liver; the explanation for this phenomenon is probably the fact that the soaps produced in the process cause a large increase in activity of pancreatic lipase. The two enzymes are definitely distinct, for that from pig's liver produces d-mandelic acid in hydrolyzing ethyl dl-mandelate, whereas that from the pig's pancreas yields the l-stereoisomeride under the same conditions.

CHAPTER III

Chemistry of Enzymes and Enzymatic Reactions

In order to understand the mechanism of enzymatic reactions a number of fundamental principles underlying chemical reactions must first be discussed. It should be emphasized, however, that enzymes rarely behave in strict accordance with the various chemical formulae and equations. Most of the reactions are very complex, and the formulae suggested merely express the processes in a general way.

1. Electrolytic Dissociation. Many of the enzymes behave like amphoteric substances, possess certain well-defined chemical and physical properties and have an isoelectric point at which the negatively charged anions and positively charged cations of the molecule are present in equal concentration.[256,423,827,835,1158] Invertase, for example, is an amphoteric substance, the acid dissociation constant of which is $10^{-6.7}$ or 2×10^{-7} and the base dissociation constant about 10^{-12}. Invertase travels to the anode in a weakly acid solution and is well adsorbed by electropositive but not by electronegative adsorbents. It is probable that the enzymatically active part of invertase is its undissociated portion.[406] The optimal action is at its isoelectric point which in the case of this enzyme is a wide isoelectric zone.[1158] The undissociated portion of catalase is active while the anions and cations are either inactive or possess very little activity.[560] Its isolectric point corresponds to its optimal reaction (pH 7.0).

In the case of amylase and pepsin, only the cations are active[478,904] while with trypsin, only the anions are active, and the activity of this enzyme is proportional to the concentration of the anions. Trypsin acts as a positive monovalent ion in solutions of pH 2.0 to 10.0.[901] It is probably isoelectric at pH 10.2 and behaves like a monovalent negative ion on the alkaline side of pH 10.2. Trypsin must also be a strong base since there is no evidence of any undissociated form on the acid side of pH 10.2. Further information on the electrochemical nature of enzymes may be obtained elsewhere.[409,423,923]

2. Velocity of Reactions. Enzymes influence the velocity

of chemical reactions and therefore their laws should be briefly discussed. Chemical kinetics is based upon the law of mass action, namely, that the amount of substance undergoing change in a unit of time is proportional to the active mass of the unchanged substance present during that time. The active mass of a reacting substance is calculated as mols of substance per liter of solvent. The type of reaction depends upon the number of different substances undergoing change. *Monomolecular* reactions are those in which one substance is undergoing change. If a represents the concentration of this substance, x the number of gram-molecules of it which are transformed in time t, k^1 the velocity constant, and the temperature remains constant, then the velocity of the reaction may be expressed as:

$$\frac{dx}{dt} = k^1 (a - x)$$

or, on integration,

$$k^1 = \frac{1}{t} \ln \frac{a}{a - x}$$

If k is substituted for $0.434\, k^1$ (in order to change from the natural to the decimal logarithms: when the decimal log is used, the value of the constant is multiplied by 0.434),

$$k = \frac{1}{t} \log \frac{a}{a - x}$$

and for any particular period of the reaction,

$$k = \frac{1}{t_2 - t_1} \log \frac{a - x_1}{a - x_2}$$

x_1 and x_2 are the amounts of substance changed after t_1 and t_2. The curve thus obtained is a "logarithmic curve."

The constant k has been determined for a number of reactions. If half of the substrate (a) has been changed, then:

$$x = \frac{a}{2},$$

or

$$k = \frac{1}{t} \log 2 \text{ or } t = \frac{\log 2}{k}$$

Bimolecular reactions are those in which two substances are undergoing change simultaneously. The amount of change at any moment is proportional to the product of the active masses (or concentrations) of the substances undergoing change. If the initial quantities of the two reacting substances (a) and the amounts of each (x) that have undergone change are the same, then the velocity of the reaction may be expressed as:

$$\frac{dx}{dt} = k(a - x)^2,$$

or, on integration,

$$k = \frac{x}{t(a - x)a}$$

If the initial amounts are different (a, b) but x is the same, then:

$$\frac{dx}{dt} = k(a - x)(b - x)$$

or

$$k = \frac{1}{(a - b)t} ln \frac{(a - x)b}{(b - x)a}$$

A bimolecular enzyme reaction is illustrated by the destruction of *B. coli*-agglutinins by trypsin.[50]

The velocity of a *trimolecular* reaction may be expressed as:

$$\frac{dx}{dt} = k(a - x)^3$$

or, on integration,

$$k = \frac{1}{t}\frac{x(2a - x)}{2a^2(a - x)^2}$$

The value of k is characteristic for a given reaction at a given temperature, but changes if the conditions which affect the reaction or its mechanism are altered.

The most carefully investigated enzyme reactions are of zero order, the transformed quantity of substrate being proportional only to the time in which the reaction takes place.[51] This law depends upon the presence of a great excess of substrate compared with the amount of enzyme. If the substrate is not present in excess, if the enzyme is destroyed, or if the products of the reaction exert an influence, departures from the above law will occur.

If one of two reacting substances is present in considerably larger concentration than the other, as in the case of the inversion of cane sugar by acids,[1272] in which both sugar and water are interacting but the latter is present in excess, the reaction is considered to be of the first order and the concentration of the water is considered as constant. Only one molecule, that of the sugar, therefore, is regarded as undergoing a change. The reaction velocity, as expressed by the constant k, is proportional to the concentration of the dissociated portion of the acid, which is the catalyst, or to the hydrogen ion concentration. This relation is particularly true at high dilutions of the acid.[50,808]

TABLE III

The rate of inversion of cane sugar by invertase[581]

TIME (t)	ROTATION OF INVERTASE-CANE SUGAR MIXTURE*	VELOCITY CONSTANT $k = \frac{1}{t} \log_{10} \frac{a}{a - x}$
minutes	*degrees*	
0	+24.50 (R)	—
30	+14.27	0.00558
60	+7.90	0.00530
90	+3.00	0.00539
110	+0.8	0.00534
130	−1.49 (L)	0.00559
150	−2.40	0.00533
∞	−7.47	—

* Alkali has been added to destroy the invertase and to stop the mutarotation.

TABLE IV

The rate of inversion of cane sugar by invertase[826]

TIME (t)	CANE SUGAR (a)	INVERT SUGAR (x)	VELOCITY CONSTANT (k)
minutes			
0	+4.334	—	—
0.5	4.324	0.010	—
21.0	3.945	0.389	0.00145
60.0	3.260	1.074	0.00151
130.0	2.129	2.205	0.00164
190.2	1.330	3.004	0.00171
246.0	0.744	3.590	0.00176

The kinetics of the catalytic action of enzymes is not as simple as that of mineral acids. The kinetics of the hydrolysis of cane sugar by invertase has been studied most extensively. The results of experiments,[581] in which a 10 per cent solution of cane sugar was incubated at 30°C. with about one-fourth its volume of pressed beer yeast solution, are illustrated in Table III. However, it was later found that the values of k increased with the time of incubation, as shown by the results obtained at pH 4.7 and 22.3°C. with an acetate mixture $\left(\frac{N}{50}\right)$ as a buffer (Table IV). In the case of other enzymatic reactions the velocity constant decreases as the time of incubation is prolonged as is illustrated in the hydrolysis of lactose by lactase:[41]

TIME	VELOCITY CONSTANT (k)
hours	
1	0.0640
2	0.0543
3	0.0460
5	0.0310
24	0.0129

The best means of comparing the reaction velocities of enzyme processes is by using the time required for a definite amount of change as a measure of this velocity.[105,186] Since the reaction of hydrolysis is always accompanied by that of synthesis, the actual velocity of a reaction at any moment is equal to the difference between the velocities of the hydrolytic and synthetic processes.

The mechanism of the hydrolytic action of invertase is said to involve the formation of a compound between the enzyme and the sucrose. This is followed by the decomposition of the compound into the enzyme and hexoses. The formation of intermediate addition compounds has been proved for a number of enzymatic reactions. The influence of inhibiting substances on enzymes is explained as a combination of the former with the latter preventing thereby a combination between the enzyme and the substrate.[423]

The reaction velocity of invertase at 37°C. has been studied at different hydrogen ion concentrations.[874] At the optimum, pH 4.5, the value of k, using the monomolecular reaction, increases as the reaction proceeds. At a lower acidity (>pH 4.5), the value of k is almost constant. In some cases, decreasing values of k are obtained, due probably to the destruction or inactivation of the enzyme. In other words, the reaction proceeds according to the monomolecular law only under certain conditions.[369,404,923]

According to the *Schütz rule,*[1108] the amounts of protein digested by different concentrations of pepsin are proportional to the square root of the concentration of the enzyme, i.e., if x is the amount of protein digested, E the enzyme concentration and p the proportionality factor:

$$x = p\sqrt{E}$$

This rule is only valid at the beginning of the reaction, or until 30 to 50 per cent of the substrate has been decomposed. The reaction velocity is proportional not only to the amount of undecomposed substrate, but is also inversely proportional to the quantity already decomposed.[48–51] In some enzymatic reactions, the amount of digestion is proportional to the square root of the time during which the reaction takes place:

$$x = q\sqrt{t}$$

3. Combination of Enzyme and Substrate. During enzymatic reactions the enzyme and substrate form a combination which is considered by some[897] as a chemical phenomenon, obeying the laws of kinetics in homogeneous solutions, and by others[109] as an adsorption phenomenon. It has been found in invertase experiments that the enzyme-substrate compound is very labile and readily breaks down into the free enzyme, dextrose and levulose.[828] The fact that invertase combines with the substrate may explain why the velocity of this reaction is not monomolecular.[1213] However, the formation of an intermediate compound between the enzyme and substrate will not account for the facts that the addition of gelatin does not affect the rate of hydrolysis of a casein solution by trypsin, and that if the enzyme is heated with either gelatin or casein its ability to split the other protein is protected from heat inactivation.[902]

4. Equilibrium. Very few chemical reactions proceed in one direction only; the products formed tend to recombine into the original substances. These reverse reactions proceed with their own velocities. Equilibrium is attained when the two reactions proceed in the opposite directions at such a rate that the concentrations of the reacting substances remain unchanged. An illustration

commonly given is the equilibrium established between an acid, alcohol and ester in the presence of lipase:

$$\text{ester} + \text{water} \rightleftarrows \text{acid} + \text{alcohol}$$

Starting with an ester, water and lipase, the reaction of hydrolysis is at first rapid while the reverse action, namely of ester synthesis is at first zero. As soon as appreciable amounts of acid and alcohol are formed, synthesis begins and gradually increases as the concentration of the products of hydrolysis becomes greater. The hydrolytic reaction, on reaching its maximum, begins to decrease as the concentration of the substrate diminishes, until a point is finally reached at which the amount of ester synthesized is just equal to the amount of ester hydrolyzed. The rates of velocity in opposite directions then become equal and the reaction is at equilibrium.

The equilibrium is dynamic, since as much of the original substance is decomposed as is synthesized. The velocity coefficient of the reaction proceeding to the right may be expressed as k_1, that of the reaction proceeding to the left as k_2, the concentration of the substrate as a_1, that of the end-products as a_2, the number of different active molecules of the reactions proceeding to the right and left as n_1 and n_2 respectively, and the amount of change in time t as x. The two reactions in opposite directions may then be represented by:

$$\frac{dx}{dt} = k_1\,(a_1 - x)^{n_1} \quad \text{and} \quad \frac{dx}{dt} = k_2\,(a_2 - x)^{n_2}$$

then, at equilibrium:

$$k_1\,(a_1 - x)^{n_1} = k_2\,(a_2 - x)^{n_2}$$

or

$$\frac{k_1}{k_2} = \frac{(a_2 - x)^{n_2}}{(a_1 - x)^{n_1}} = k$$

or the equilibrium constant at a definite temperature.

The value of k varies with the temperature, since k_1 and k_2 do not necessarily change at the same rate with the change in temperature.

In experiments on the action of lipase upon iso-amyl alcohol and normal butyric acid the position of equilibrium is the same whether one starts with an ester and water or a mixture of alcohol and acid.[326] Differences in the activity or in the amount of lipase do not influence the position of equilibrium. Differences in the concentration of the substrate as well as in the amount of water present, however, influence the velocity constants.

5. SYNTHETIC ACTION OF ENZYMES AND FALSE EQUILIBRIUM. Enzymes accelerate both hydrolytic and synthetic processes. If equilibrium has been established at any point in the reaction, except when a complete change in one direction has taken place, the enzyme will produce hydrolysis and synthesis at the same time, as for example the equilibrium between methyl acetate, water, acetic acid and alcohol. Theoretically, all reactions are reversible and the apparent irreversibility of some reactions may be due merely to the limitations of the methods, or to improper conditions. Considerable synthesis may take place if the equilibrium point is near complete hydrolysis or if the synthetic product is deposited in an insoluble form, removed, or changed by some independent reaction.

The synthesis of ethyl butyrate from dilute butyric acid and ethyl alcohol by lipase (pancreatic extract) occurs, in the presence of antiseptics, at ordinary temperatures. The ester can readily be separated by distillation and decomposed again by the same enzyme.[643] Hydrolysis of an ester by means of lipase does not proceed as far as that by means of hydrochloric acid, due perhaps to the formation of a compound between the enzyme and substrate or between the enzyme and end-products.[326] The concentration of water greatly influences the proportions of alcohol, acid and ester at equilibrium.

The synthesis of carbohydrates and glucosides by enzymes was first established by Croft Hill.[563] However, the product of the synthesis was not the same as that which was originally hydrolyzed by the enzyme, but was its isomeric form. Maltase acting upon a concentrated solution of dextrose produced iso-maltose. This is a disaccharide which can be hydrolyzed by emulsin, but not by maltase, into two molecules of dextrose. It was, therefore, suggested that an enzyme could synthesize only those substances which it could not hydrolyze.[41] It is likely that the formation of iso-maltose was a result of the action of emulsin, which was present in the crude brewer's yeast used by Croft Hill. It is also possible that the same enzyme may act on both optical isomers at different rates.[109]

Pepsin solutions can synthesize proteins from the products of the peptic digestion of casein.[1027] It is possible that this is not an enzymatic phenomenon, but a colloidal precipitation, produced by certain preparations of pepsin.[107] However papain, pepsin and rennet can form the synthetic product "plastein" from solutions of peptone[295,552] and trypsin can synthesize a protamine.[1191] Soy bean urease is another synthesizing enzyme.[654] Of special interest in this connection is the enzyme carboligase, which is present in yeast juice and is capable of building up carbon chains; benzaldehyde and pyruvic acid are condensed to form l-acetyl phenyl carbinol ($HO \cdot CH \cdot C_6H_5 \cdot COCH_3$).[887]

The possibility of the synthesis of an optically active substance from inactive constituents by an optically active enzyme is suggested by the report of an asymmetric synthesis of optically active benzaldehyde-cyanhydrin by the action of emulsin upon a mixture of hydrocyanic acid and benzaldehyde.[1052] A large number of β-glucosides of various primary alcohols have been prepared by the use of emulsin and also certain α-glucosides by the use of yeast maltase.[170] These syntheses represent true equilibria, since the same point is reached regardless of whether the reaction is started with one or the other end-product, as for example, dextrose or the disaccharide in Croft Hill's experiments. The same observation is true in the case of β-ethyl-glucoside and emulsin,[170] β-glycerol-glucoside[107] and in the action of castor oil bean lipase.[608]

However, in addition to true equilibria, false equilibria have also been observed. As pointed out above, the equilibrium position produced by an acid (inorganic catalyst) is usually different from that caused by an enzyme. It is possible that, in the case of an enzyme, a portion of it remains undecomposed at the end of the reaction and that this portion becomes a part of the chemical system at equilibrium.[16] The equilibrium which may be established as a result of the injurious action of the products of the reaction upon the enzyme is not a true equilibrium. By removing the products or by diluting the system, the reaction may be made to recommence. Bayliss[109] called this phenomenon "false equilibrium." It occurs more frequently when very small amounts of enzyme are used. The addition of fresh amounts of enzyme to a reaction in a seeming equilibrium will renew hydrolysis.

6. Energy Transformation in Enzymatic Reactions. The heat liberated in enzymatic reactions, especially those of hydrolysis, is very small as is shown by subtracting the heat of combustion of the end-products from that of the substrate (Table V). During the decomposition of proteins by enzymes practically no heat is liberated.[489] Enzymes, however, influence the energy changes in the cell.[1060]

7. Heterogeneous Systems. If substances which act upon one another are not mixable homogeneously in the form of gases, liquids or solutions, but belong to different phases, the reaction process may be different than that of homogeneous systems. The phenomena considered up to the present have been those of homogeneous systems. Enzyme reactions, however, often occur in heterogeneous systems, especially since enzymes are colloids.[923] The reaction may take place when the enzyme is in the solid phase. It has actually been found that, in some cases at least, enzymes will act even if not in solution. Reactions in heterogeneous systems proceed very nearly in the same manner as in homogeneous systems, especially if diffusion is unimpaired (as in the case of Mett's tubes).[48] For example, invertase adsorbed on charcoal or aluminium hydroxide exerts the same effect as invertase in solution.[873] In fact, the rate of the reaction may be increased. This increase is due to the concentration of the active mass of the substances undergoing change upon the surface of the enzyme. The phenomena of adsorption are discussed in detail elsewhere (see pp. 34–36).

TABLE V

The theoretical amount of heat liberated in enzymatic reactions[560]

SUBSTRATE	END-PRODUCTS	CALORIES LIBERATED (SUBSTRATE — PRODUCTS)
Ethyl butyrate	Ethyl alcohol + butyric acid	1.2
Maltose	Glucose	3.8
Cane sugar	Invert sugar	4.5
Salicin	Salicyl-alcohol + glucose	5.3
Urea	Ammonia + CO_2	4.3
Hippuric acid	Glycocoll + benzoic acid	6.5
H_2O_2	$H_2O + O_2$	21.7

8. Colloidal Nature of Enzymes. All enzyme preparations, which have been analyzed, with the exception of yeast invertase, have been found to contain nitrogen, ranging from 13.8 per cent for pepsin[302] to 17.1 per cent for castor bean lipase.[420] Preparations of yeast invertase have been obtained which contained less than 1.3 per cent nitrogen.[872] Chemical analyses of enzymes, with the exception of yeast invertase which also contains a carbohydrate phosphate complex, resemble those of pure proteins. These proteins, which form the enzymes or are closely associated with them,

are amphoteric, forming salts with acids and bases. Most enzymes are, therefore, either themselves in a colloidal condition or are closely associated with substances having colloidal properties.

Enzymes form colloidal solutions in water, which are actually suspensions of ultra-microscopic particles, or "emulsoids," possessing a low diffusibility. As a result, enzymes possess the various properties characteristic of colloids, as for example, an enormous surface development and the power of adsorption. Many colloids possess positive or negative electric charges, or will give or take up such charges as a result of the action of electrolytes. They are therefore capable of forming salts with acids or alkalies. Amphoteric substances, such as proteins, to which most enzymes are probably related, are charged positively or negatively depending upon the ion with which they are combined or upon the reaction of the solution in which they are suspended. The colloid particles travel towards the pole of opposite sign to that of their charge. However this electric transference of enzymes may be due to accompanying impurities.[1281]

As a result of adsorption, colloids will take up various other substances, including colloids, and form "adsorption compounds." This is especially true if the substances have charges of the opposite sign. The neutralization of electric charges may be the cause of precipitation of colloids, while, in some cases, the repulsion of similarly charged particles may be responsible for the permanency of a solution. Invertase is adsorbed quantitatively by electropositive adsorptive agents, such as $Fe(OH)_3$ and $Al(OH)_3$.[821] Since invertase is adsorbed by these substances, both in acid and alkaline solutions, it has been suggested that this enzyme acts as an acid. Pepsin is also of an acid nature, since it is adsorbed by infusorial earth, and not by kaolin, while zymase is a neutral substance and is not adsorbed by $Fe(OH)_3$. Charcoal will completely withdraw lipase both from acid and alkaline solutions. Trypsin is adsorbed by a number of substances, such as silk, wool, cotton, paper, agar-agar, asbestos and glass wool.[212] Charcoal will adsorb trypsin, rennet, invertase and other enzymes. Some enzymes thus behave as ampholytes, as for example, trypsin and malt amylase, which are completely adsorbed at an acid or neutral reaction by kaolin and infusorial earth, but incompletely or not at all at alkaline reactions. In studies on the effect of charcoal upon trypsin, in which the activity of the enzyme was measured by the changes produced in the viscosity of gelatin solutions, it was found that the amount of trypsin removed from solution by the charcoal depended upon the order in which the solutions were mixed; if the trypsin and charcoal were mixed first, a very much greater amount of the enzyme was combined than if the charcoal was first mixed with the gelatin. Charcoal which had previously been treated with gelatin did not remove trypsin from solution.[898] Fractional adsorption has been used to separate various enzymes from each other[543] and for the purification of enzymes.[416] The enzymes may be removed from the adsorbent either by water or a proper agent, i.e., trypsin may be removed from charcoal by the addition of casein.

In an adsorbed condition enzymes may be as active as in a free condition;[821] the activity of invertase is the same whether the enzyme is adsorbed by a solid, such as charcoal, or by a colloid like saponin, serum, or egg albumin, distributed uniformly throughout the solution of the substrate.[873] The adsorption of enzymes, which is a result of their electrochemical properties should be differentiated from the specific combination of enzymes with their substrates or with reaction products.[822]

Many of the phenomenona of enzymatic activity can best be explained by adsorption, i.e., the rapid attainment of equilibrium owing to the concentration on the surface of the enzyme;[109] the formation of adsorption compounds between substrate and enzyme; the influence of the concentration of the substrate; the influence of the concentration of the enzyme; the action of enzymes in an insoluble condition; the dispossession from the surface through the presence of a substance more highly adsorbed; the protection from heat, and the effects of dispersion. A condensation of dissolved substances occurs at the interface between a solvent and the solid particles suspended in it and as the reaction between invertase and cane sugar apparently depends on the contact of two phases[873] the rate of inversion of cane sugar is determined by the amount adsorbed by the enzyme.[874]

These facts have a bearing upon the relation of enzymes to paper, clay and parchment filters. A Chamberland filter adsorbs lipase, amylase, pepsin and trypsin, but allows the passage of maltase, malt protease and stomach lipase. The reaction of the solution is one of the factors upon which filtration depends. At pH 6.7, Chamberland filters do not adsorb pepsin, emulsin, invertase and catalase and these enzymes are transmitted, but, at pH 3.4, the enzymes do not pass through the filter.[570]

In attempting to dialyze enzymes by means of parchment and collodium sacks, the principles of adsorption must be considered. Parchment filters retain pepsin and peroxidase and transmit invertase, diastase and other enzymes while collodium sacks retain pepsin and invertase and transmit emulsin, lactase and trypsin.[404]

There are some enzymes (rennet and pepsin) which are inactivated by shaking.[1092, 1119] The inactivation of rennet by simple shaking occurs in dilute solutions; the lower the concentration of the enzyme and the higher the speed of shaking, the greater is the injury. The presence of electrolytes and small amounts of acid (0.5 cc. $\frac{N}{10}$ HCl in 100 cc.) reduces the amount of inactivation but this effect is not a result of the hydrogen ion concentration. The strength of diastatic enzymes is also reduced by shaking, but inactivation does not occur. This reduction is a result of adsorption for a large part of the enzyme passes into the foam. The presence of saponin prevents this phenomenon.

Further information on the colloidal properties of enzymes, the influence of colloids on enzymes and the relation of the activity of enzymes to the surface tension of the medium may be obtained elsewhere.[109, 111, 297, 386, 974]

CHAPTER IV

FACTORS WHICH INFLUENCE ENZYMATIC REACTIONS

1. **Temperature.** The velocity of enzyme reactions is accelerated as the temperature is increased until a certain optimum is reached. On further increasing the temperature the reaction velocity begins to diminish until it ceases completely. It is generally believed that each enzyme has an optimal temperature characteristic of that enzyme. This optimum is, however, greatly influenced not only by the type of enzyme, but also by its concentration, the nature and concentration of the substrate upon which the enzyme acts, the reaction of the medium, the presence of activating and paralyzing substances, etc. For example, the optimal temperature for pepsin is not constant, but depends upon the reaction and period of action: at pH 1.6 and with an excess of the enzyme, the optimum is at 45° to 50°C. when the period of action is one hour; 52°C. is the optimum for a period of action of five minutes and 56° to 60°C. for a period of one minute.[367] The optimal temperature for the action of the salicinase of sweet almonds and of the maltase of *Asp. oryzae* is independent of the concentration of substrate and enzyme. Since it is usually impossible to reproduce experimental conditions exactly, one can never be sure whether a difference in the observed optimal temperature is due to a difference in the nature of the enzyme or to the experimental conditions. This optimum usually falls between 40° and 50°C. (Figs. 2 and 3) but for such enzymes as papain and bromelin, the optimal temperature is at 60°C.[270–272]

At the optimal point, the auto-inactivation of the enzyme, which proceeds slowly at lower temperatures, is increased until it balances the accelerating effect produced by the rise in temperature. Temperatures above the optimum lead to the rapid inactivation of the enzyme. In addition to the direct inactivation of the enzyme, the retarding influence of high temperatures may be due to the preferential acceleration of the resynthesis of the substrate, i.e., accelerating the reverse reaction at a greater speed than the original reaction. In other words, a shift in equilibrium may take place.[1027]

a. The temperature coefficient and temperature constant of enzymatic reactions. At temperatures below the point at which the enzyme is rapidly destroyed, an increase of 10°C. increases the velocity of enzymatic reactions (or value of K) two to three times. The relation between reactions at two temperatures 10°C. apart is designated as the temperature coefficient. It may be expressed as $K_{t \times 10}:K_t$ and is best measured at a range in which the destruction of the enzyme is least. The temperature coefficient is affected by the influence of temperature upon (1) the concentration of active enzyme, (2) the reaction velocity of the substrate-enzyme combination and (3) the position of equilibrium.[404] The following formula has been developed for the temperature coefficient in which K_1 and K_2 are reaction constants at absolute temperatures T_1 and T_2, R is the gas constant and e is the basis of natural logarithms:[50]

$$\frac{K_2}{K_1} = e^{\frac{A\,(T_2 - T_1)}{R \cdot T_1\,T_2}}$$

or

$$A = \frac{\log K_2 - \log K_1}{0.4343} R \cdot \frac{T_2 T_1}{T_2 - T_1}$$

The temperature constant (A) of enzymatic reactions usually changes with the temperature. In the action of invertase on cane sugar, A is equivalent to 10,000 at 0° to 20°C., 9,400 at 20° to 30°C. and 6,700 at 30° to 40°C.[403] A formula, $A = 11{,}400\ (1–0.009t)$, has been suggested for calculating the variation of A with the temperature in the case of invertase.[404] The temperature constants for reactions brought about by inorganic and organic catalysts are quite different; for the hydrolysis of cane sugar by HCl, A is 25,000 and for the hydrolysis by invertase 9400.[404] In the liquefaction of starch by diastase, A is practically constant between 20° and 40°C. At 55°C. this enzyme is destroyed in one hour.

A series of figures for the temperature coefficients and constants of various enzymes are illustrated in Table VI.

Further information on the temperature coefficients of the enzymes concerned in the liquefaction of starch, especially in connection with their thermostability, is given elsewhere.[388]

b. Influence of temperature upon the stability of enzymes (thermostability). At temperatures below 45°C. most enzymes in solution are more or less stable but above 50°C., the inactivation rapidly increases with an increase in temperature (Fig. 4), except for the thermoregeneration exhibited by sucrase.[130a] Enzymes differ in their thermostability, urease being one of the most resistant exceeded only by myrosin, papain and the gelatinase of *B. prodigiosus*.[499,754,1213] The activity of *Phaseolus* diastase is reduced at 40°C. and destroyed at 55° to 60°C.[1147] Invertase from different yeasts is sensitive in different degrees, the invertase of one variety being inactivated to one-half of its original value when kept for one hour at 59°C. and pH 4.5.[403]

In solution the majority of enzymes are rapidly destroyed at 70° to 80°C., although the proteolytic and oxidizing enzymes of *B. prodigiosus* and the peroxidase of bacterial cultures can resist even 100°C. for a short period.[1165] When dried, however, enzymes withstand 100° to 120°C. and even higher temperatures. The reaction of the medium greatly influences the thermostability of an enzyme, maximal stability occurring at the optimal reaction. For example, the maximal stability of invertase is at pH 4.5 which is its optimal reaction.[403,582] The peptolytic enzymes of hemolytic streptococci are destroyed at pH 7.0 in a buffered solution after ten minutes at 57°C., and at pH 5.0 the enzymes slowly deteriorate at 37°C. At pH 7.0 the invertase of streptococci is destroyed after ten minutes at 52°C., and at pH 5.0 after six hours at 37°C.[1167] Lipase resembles invertase in its susceptibility to heat and is completely destroyed after ten minutes at 55°C. The rôle of the reaction of the medium

TABLE VI

Temperature coefficients and constants of various enzymes[404]

ENZYME	SUBSTRATE	TEMPERATURE RANGE	TEMPERATURE COEFFICIENT $K_{t+10}:K_t$	TEMPERATURE CONSTANT (A)	REFERENCE
		°C.			
Lipase	Triacetin	18–28	2.6	16,700	1191
Invertase	Cane sugar	0–20	2.0	10,500 ±300	403
Diastase	Starch	30–40	2.0	12,700	657a
Rennet	Milk	20–30	2.1	13,400	1218
Trypsin	Witte peptone	15–25	2.3	14,300	1219
Erepsin	Witte peptone	15–25	2.6	16,400	1219
Urease	Urea	30–40	3	20,000	1213
Catalase	H_2O_2	0–10	1.5	6,200	1116
Zymase	Sugar	15–25	2.8	18,000	560

in determining the optimal temperature for enzymatic activity has been carefully studied in the case of the maltase of *Asp. oryzae*.[273,407]

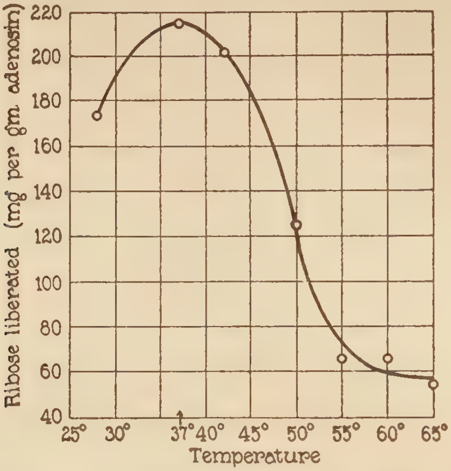

Fig. 2. Influence of Temperature upon the Action of an Enzyme (Nucleosidase)[724]

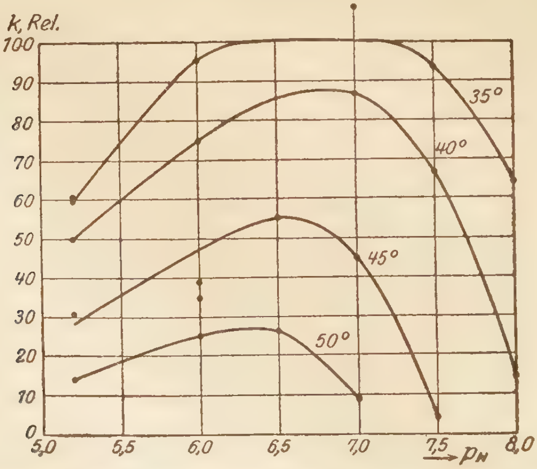

Fig. 3. Influence of Temperature and Reaction upon the Action of Plant Amylase[1147]

A certain protective action against the inactivating effect of high temperatures is exerted by the presence of proteins and impurities acting as protective colloids or buffers, and of various salts such as phosphates and chlorides in the case of amylase, which probably form heat resisting compounds with the enzyme.[1219,1281] The products of the reaction and the substrate also protect invertase and other enzymes against heat inactivation, 4 per cent cane sugar causing 40 per cent protection against heating at 60°C. for one hour.[403,581,933]

In some instances a reactivation has been found to occur when heated enzymes were allowed to stand in solution.[129,491,755,843] A solution of *Asp. oryzae* diastase, which has been heated to 95°C., then cooled and allowed to stand at room temperature, will regain its activity in five days. Similar observations have been made on oxidizing enzymes as well as on invertase and trypsin. Heat inactivated lipase may be partially reactivated by manganese salts.[419] This phenomenon is not clearly understood.

The course of the inactivation of an enzyme which results from the influence of temperature is, in the case of invertase, a monomolecular reaction and is independent of the substrate and products of the reaction.[404] If F represents the original concentration of the enzyme, y the concentration of the enzyme destroyed in time t, and K_c the constant of inactivation, then:

$$\frac{dy}{dt} = K_c\,(F - y)$$

or

$$K_c = \frac{1}{t} \ln \frac{F}{F - y}$$

If a is the original concentration of the substrate, then:

$$\frac{dx}{dt} = k\,(a - x)\,(F - y)$$

or the velocity of the reaction is proportional to the product of the concentration of enzyme and unchanged substrate.

The phenomenon of inactivation was first studied in connection with emulsin, with salicin as a substrate.[1188] The K_c was more or less constant at 60°, 65°, and 70°C., while at 40° and 50°C. it greatly decreased with time. Further studies on inactivation have been carried out on blood catalase, rennet, pepsin and trypsin.[48,50,898,1116] The K_c for rennet at 47° to 60°C. is 0.0386 and for pepsin at 66.8°C. is 0.0305. If the ability of trypsin to liquefy gelatin is used as an index, the concentration of the enzyme has no influence upon the velocity of inactivation, and 0.010073 is the value of K_c at 67.15°C.

In view of the fact that it is very difficult to determine definitely the destructive temperature for an enzyme, which depends on the K_c and the length of heating, Euler[404] to avoid confusion, defined the "destructive temperature" as "that temperature, at which the enzyme loses one-half of its activity, when heated, in an aqueous solution (free from substrate), for sixty minutes at a definite optimal hydrogen ion concentration." Further information on the influence of temperature upon enzyme activity may be obtained elsewhere.[403,834,1178]

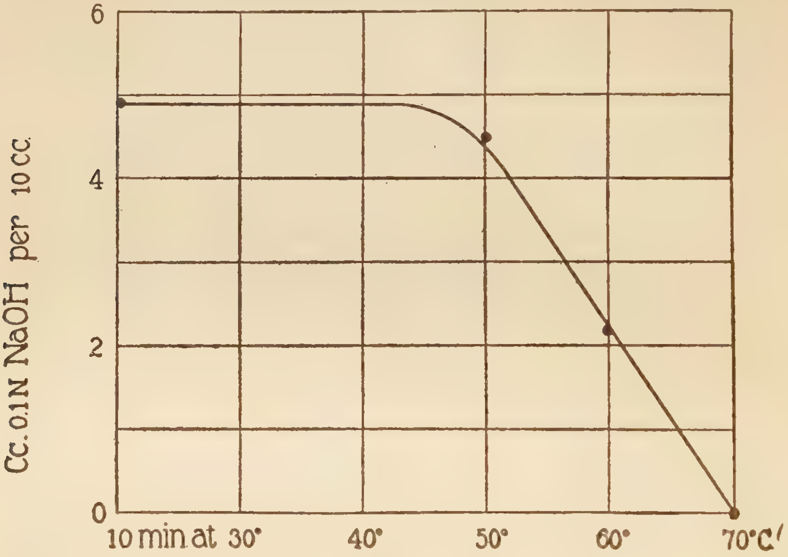

FIG. 4. HEAT STABILITY OF AN ENZYME: INTRACELLULAR LIPASE OF PNEUMOCOCCUS[56]

2. CONCENTRATION OF SUBSTRATE. Although the velocity of the hydrolysis of cane sugar by acids is constant and is proportional to the amount of unchanged substrate, the velocity of the hydrolysis of cane sugar by invertase as well as the rate of other enzymatic reactions may increase or decrease as the concentration of the substrate is raised. If the amount of the substrate is very small in comparison with the concentration of the enzyme an increase in the concentration of the substrate may increase the velocity of the reaction. For example, the velocity of inversion is increased by increasing the sucrose concentration up to 4 or 5 per cent.[874] Above that concentration, the relative amount of sugar decomposed de-

creases with an increase in the concentration of sugar[581] as shown in Table VII. In other words diluting a concentrated substrate to 4 or 5 per cent increases the proportion of it which will be digested. The data in Table VII also demonstrate that the reaction velocity is proportional to the concentration of the enzyme. With colloidal substrates, as in the action of trypsin on gelatin, an increase in the concentration of the substrate may result in a decrease in the rate of hydrolysis, due either to the influence of viscosity or to the effect of the products of hydrolysis.[109]

3. Concentration of Enzyme. The methods used for estimating the strength of enzymes are often based upon the fact that the velocity of an enzyme reaction is an index of the concentration of the enzyme. In most instances a direct linear relationship has been found between the velocity of the reaction and the amount of enzyme used. The time required by invertase to cause a certain amount of inversion, for example 40 per cent, is directly proportional to the concentration of the enzyme.[874] Similar results have been reported for urease,[1213] trypsin, pepsin[900] and amylase.[304] This linear relationship is explained by the assumption that the enzyme and substrate form a combination which contains the only portion of the substrate which undergoes hydrolysis.[404,550,828] The rate of change of the substrate is, therefore, directly proportional to the concentration of the enzyme.

TABLE VII

Influence of the concentration of invertase and of substrate upon the rate of inversion of cane sugar at 30°C.[581]

INVERTASE CONCENTRATION (*E*)	TIME OF ACTION (*t*)	ENZYME CONCENTRATION × TIME (*E* × *t*)	PER CENT OF INVERSION OF SUBSTRATES (CANE SUGAR) OF DIFFERENT CONCENTRATIONS		
			45.5 grams per liter	90.9 grams per liter	273 grams per liter
	minutes				
2.00	15	30	73.2	45.3	11.2
2.00	30	60	93.0	74.2	22.0
1.50	20	30	73.2	44.8	11.2
1.50	40	60	92.8	74.5	22.7
1.00	30	30	72.9	45.3	11.5
1.00	60	60	93.0	74.7	22.3
0.50	60	30	72.9	45.2	11.4
0.50	120	60	92.7	74.5	22.6
0.25	120	30	73.1	45.2	10.9
0.25	240	60	92.7	74.7	21.9

With certain other enzymes, however, the velocity is proportional to the square root of the concentration of the enzyme—the so-called Schütz and Borissov law.[1108] Both of these relationships may be correct if the relative concentrations of enzyme and substrate, the type of enzyme, or the different stages of the reaction are taken into consideration.[109] The linear relation seems to hold when the amount of enzyme is much smaller than that of substrate, so that all of it can combine with the latter. With a relative increase in the concentration of enzyme, the law of Schütz and Borissov is apparently fulfilled.[510]

4. End-Products of the Reaction. In many cases, the products of the reaction interfere with the action of the enzyme and as a result the concentration of active enzyme changes during the course of the reaction. The amount of active enzyme is not always directly proportional to the total enzyme concentration. For example, the peptone formed as a result of the action of pepsin upon the substrate combines with the pepsin and renders it inactive.[897] The total concentration of the enzyme and the active concentration are no longer equal nor proportional and since the rate of the reaction is proportional to the active concentration of enzyme, it is not proportional to the total concentration. The pepsin and peptone are believed to combine according to the law of mass action:

$$\text{Pepsin} + \text{peptone} \rightleftarrows \text{pepsin-peptone}$$

$$\frac{C\ \text{pepsin} \times C\ \text{peptone}}{C\ \text{pepsin-peptone}} = k$$

If E represents the total enzyme concentration, Q the concentration of the active (uncombined) pepsin, d the concentration of peptone at the beginning of the reaction, x the amount of peptone formed during time t, k the equilibrium constant and A the active concentration of substrate present at the beginning of the reaction, then:

$$\frac{Q\,[d - (E - Q) + x]}{E - Q} = k$$

or

$$\frac{dx}{dt} = kQ(A - x)$$

Pepsin thus enters directly into the equation and cannot be considered a catalyst, in the sense usually defined. Since it combines with one of the products of the reaction, its presence necessarily affects the equilibrium point. The reaction can be regarded as a special type of a bimolecular reaction in which one of the reacting substances (the enzyme) forms a highly dissociated compound with one of the products. The optimal hydrogen ion concentration for the combination between pepsin and protein corresponds to the optimum for hydrolytic action.[896]

The retardation of the reaction velocity of invertase by the products of the reaction (fructose to a greater extent than glucose) is also probably due to the formation of chemical compounds between the enzyme and these products of the reaction.[828]

5. REACTION OF THE MEDIUM. Most enzymes are greatly influenced by the reaction of the medium in which they act. There is an optimal hydrogen ion concentration for the activity of each enzyme, and there are also upper and lower limits of reaction, above or below which the enzyme is inactive or may even be rapidly destroyed. This phenomenon was observed by the earlier investigators[436,933] of enzyme reactions long before the exact rôle of the hydrogen ion concentration of the medium was recognized. Among the most important contributions to this subject, is the work of Sörenson.[1157] In a study of the influence of the reaction upon the inversion of cane sugar by invertase, it was found that, at 52°C., the maximal activity was at pH 4.4 to 4.6, with a wide optimal zone from pH 3.5 to 5.0 (Figs. 5 and 7). This subject has since been studied in more detail at lower temperatures, so as to avoid the destruction of the enzyme, which occurs near the optimal temperature of 52°C., and it has been demonstrated experimentally that the amount of digestion is a function of time for any hydrogen ion concentration.[826] The optimal reaction, together with the nature of chemical change produced, has served as a basis for the specific differentiation of enzymes, as shown in Table VIII.

Enzymes of different origins, as for example the diastase from malt and that from *Asp. oryzae* even though they act upon the same group of substrates, may vary in their optimal reactions, as shown in Figure 5 and Figure 6.[1129] An even wider variation is found in the case of protease (Table IX). Both pepsin and trypsin will hydrolyze proteins in neutral solutions, although to a much smaller degree than at their optimal reaction.

The optimal hydrogen ion concentration for an enzyme reaction often depends upon the type of buffer solution used, for example, the

TABLE VIII

Optimal reactions for the activity of various enzymes

ENZYME	SOURCE	OPTIMAL pH	REFERENCE
Amylase (diastase)	*Asp. niger*	3.5–5.5	468a
	Asp. oryzae	4.8 (saccharogenesis)	1129
	Asp. oryzae	3.0 (viscosity)	786
	Cabbage, carrot, white turnip	6.0	422
	Duodenal contents (infants)	6.0–8.0 (viscosity)	1056a
	Fusarium, Colletotrichum	6.0	641
	Malt	4.4–4.5	1129
	Malt	4.3 (at 25°C.)	914
	Malt	6.0 (at 69°C.)	914
	Pancreas	7.0	1129
	Phaseolus	4.0–6.0	1147
	Potato juice	6.0–7.0	797
	Saliva	6.1–6.2	830
	Saliva	5.6 (acetate buffer) 6.6 (phosphate buffer)	505
	Urine	6.1	333
	Yellow turnip	4.0–7.0	422
Arginase	Liver	10.0	589
Autolytic enzymes	Animal tissues	4.5	182
Autolysis of yeast	Yeast	6.1	315
Bacterial enzymes	Hemolytic streptococci	7.0–7.9	1167
Bacteriophage (d'Herelle's phenomenon)	Bacteria	8.0 to 8.5	493, 302
Butyrase	Intestine of infant	8.5	298
	Pancreas, serum, human milk	8.0	298
	Stomach of infant	4.0–5.0	298
Carboxylase	Yeast	5.3–6.2	887
Catalase	Blood	7.5 (10 minutes)	151
	Liver	7.0	1158
	Vegetables	7.0–10.0	422
Cellobiase	Fungi	About 6.0	1158
Desamidase	Yeast (grown on asparagin)	7.6–8.0	406
Dextrinase	*Asp. oryzae*	4.0	786
Emulsin		4.4	693

TABLE VIII—*Continued*

ENZYME	SOURCE	OPTIMAL pH	REFERENCE
Emulsin (β-phenol glucosidase)		4.0–5.3	1278
Endoenzymes	Pneumococci	7.0–7.8	55
Erepsin	Intestine (pig)	7.9 (glycyl-glycin)	316
	Intestine (pig)	8.6 (conductivity method)	390
	Intestine (dog)	7.7 (albumose)	1034
	Ox spleen	7.5–8.5	545
	Yeast	7.8	315
Esterase	Blood serum (rabbit)	8.0	1035
	Pancreas (rabbit)	8.3–9.0	1035
α-glucosidase	*Asp. oryzae*	5.8–6.6	273, 833
β-glucosidase	*Asp. oryzae*	About 5.0	444
Inulase		3.8	995
Invertase	*Asp. niger*	2.5–3.5	639
	Potato juice	4.0–5.0	797
	Yeast	4.4–4.6 (52.1°C.)	1158
	Yeast	4.2 (22.3°C.)	826
	Fresh yeast cells	4.2–5.2	404
Lactase	Yeast	About 7.0	1280
Lipase	Blood	7.8–8.6	1032
	Castor oil	5.0	1158
	Duodenal juice	8.5	298
	Gastric juice	4.0–5.0	298
	Gastric juice of dog	4.9 (2.5 to 8.0)	1187
	Serum	7.0–8.6	
Maltase	*Asp. oryzae*	3.0 (35.5°C.)–7.2 (47°C.)	273
	Asp. oryzae	4.0	406
	Beer yeast	6.6	833
Nucleosidase	Animal tissues	7.5	724
Oxidase	Vegetables	7.0–10.0	422
Pancreatin (trypsin-erepsin)	Ox pancreas	9.7 (gelatin liquef. 37°C.)	941
	Ox pancreas	7.7–8.0 (peptone decomp.)	826
Pectase	Fruit	4.3	400
Pepsin	Animal tissues	3.0–3.5 (gelatin)	316
	Stomach	1.2–1.6 (acid albumin)	1158
	Stomach	1.4 (edestin)	832
	Yeast	4.0–4.5	315, 761

TABLE VIII—*Concluded*

ENZYME	SOURCE	OPTIMAL pH	REFERENCE
Peroxidase	Vegetables	7.0–10.0	422
Phytase	Grain	5.4–5.5	19
Protease	*Asp. oryzae*	5.1	912
	Autolyzing animal tissue	4.5	182
	Bacteria	6.0–7.0	317
	Malt	3.7–4.2	761
	Malignant human and rat tumors	7.0	422
	Papain	5.0–7.0	417, 458
α-protease	Ox spleen	8.8	545
β-protease	Ox spleen	5.4	545
Proteolytic enzymes	Yeast	6.7–8.5	11
Raffinase	Yeast	4.0–5.0	1280
Rennet (lab)	Stomach	5.0	293
	Stomach	6.0–6.4	832
Trypsin	Animal tissues	7.8 (peptone)	316
	Pancreas	9.5	1021
	Pancreas	8.3 (casein)	1128
	Pancreas	7.5–8.3 (fibrin)	751
	Yeast	7.0 (peptone)	315
	Yeast	8.0	761
Tyrosinase	Potato	6.5–8.0	1002
Urease	Soy bean	About 7.0	1214
Zymase	Living yeast	4.5–5.5 (28°C., no nitrogen)	403a
	Living yeast	4.5–6.5 (28°C., plus yeast water)	403a
Zymo-phosphatase	Yeast	6.2–6.6	403b

optimal reaction for saliva diastase is pH 6.1 to 6.2 in phosphate, acetate or sulfate mixtures and pH 6.9 in chloride and nitrate mixtures[830] and the optimal pH for pancreatic diastase is 7.2 in a phosphate mixture and 5.6 in an acetate mixture.[505] These differences, however, may either be fictitious and be caused by the difficulty of measuring accurately the reaction of different buffers or be due to the stimulating or inhibiting action of the salts comprising the buffers. The effect of salts on enzymatic reactions depends upon the hydrogen ion concentration; for example, the addition of NaCl does not influence the action of invertase at its optimal reaction, but decreases its activity at other hydrogen ion concentrations.[418] The degree of

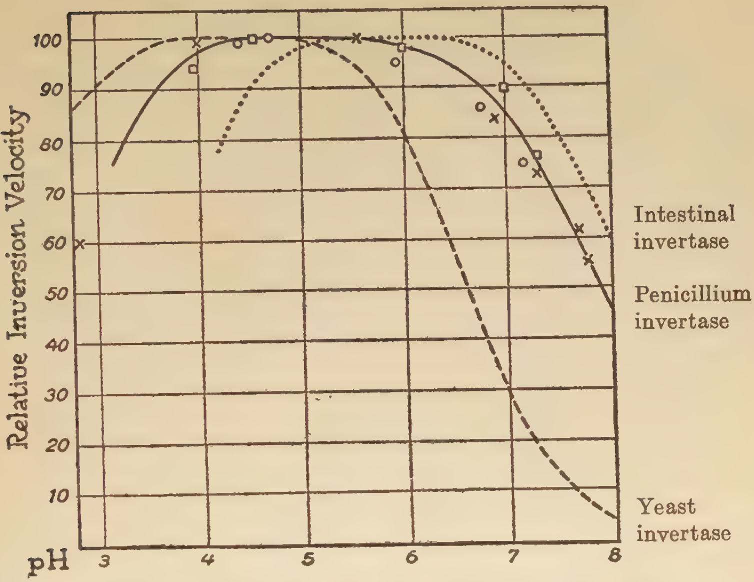

FIG. 5. INFLUENCE OF REACTION UPON THE ACTIVITIES OF THREE TYPICAL INVERTASES[413]

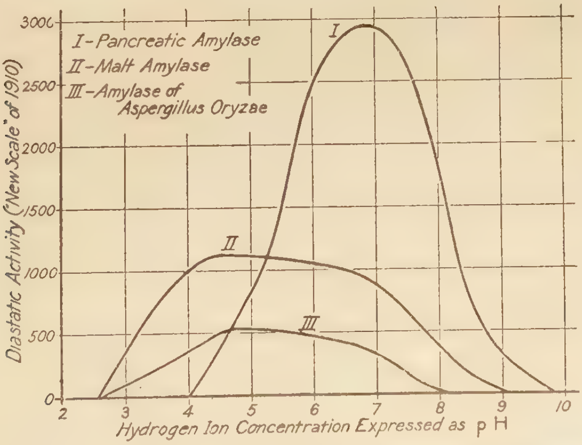

FIG. 6. INFLUENCE OF REACTION UPON THE ACTIVITY OF THREE TYPICAL AMYLASES[1129]

purification of an enzyme as well as the nature of the substrate influence the reaction optimum.[1288]

The optimal reaction for the activity of an enzyme may also depend upon the temperature at which the activity is studied. For instance at 47°C. the optimal reaction for the activity of *Asp. oryzae* maltase is pH 7.2, and at 35.5°C. pH 3.0; the optimum for malt diastase at 25°C. is pH 4.3, and at 69°C. pH 6.0.[271] However, these hydrogen ion concentrations which are different when measured at room temperature may have been similar at the temperatures of the actual experiment because of the dissociation of the buffer at the latter temperatures. When two different methods are used to measure the activity of an enzyme, especially methods in which the rate of the disappearance of the substrate and of the formation of end-products is determined (as in diastatic enzymes), distinctly different optima may be observed, and the presence of different component enzymes in the solution may be conjectured. For example, the optimal zone for the activity of *Phaseolus* diastase is pH 5.0 to 5.5, if the rate of the formation of reducing sugars is determined, but is pH 4.0 to 6.0, if the rate of disappearance of starch is measured.[1147] It is probable that the chemical changes measured are different. In place of one optimal point, it is sometimes possible to determine a broad zone of reaction at which an enzyme solution attains maximal activity.

TABLE IX

Optimal reaction for the activity of proteolytic enzymes[761]

ENZYME	SOURCE OF ENZYME		
	Higher animals	Yeast	Malt
	pH	*pH*	*pH*
Pepsin or peptase	1.5	4.0–4.5	3.7–4.2
Trypsin or tryptase	8.0	7.0	6.3

The effect of the reaction upon enzymatic activity may be due to its influence on the degree of ionization of the enzyme for in the case of certain enzymes, such as invertase, the un-ionized molecule or the "dissociation" residue contains the active enzyme; with some enzymes, pepsin for example, the cation is active, and with others, such as trypsin, lipase and maltase, the anions are the active portions.[833] The analogy between the activity curve of enzymes and the dissociation curve of weak acids and bases strongly suggests that enzymes are amphoteric substances.[739] Alkalies stimulate trypsin in direct proportion to their dissociation. However, with vegetable lipase there is no correlation between the activating action of different acids and their dissociation constants.[42] No other acids increase the potency of pepsin to the same extent as hydrochloric acid. This variation bears no relationship to the dissociation of the acids. The increase in the activity of the pepsin is probably due to the formation

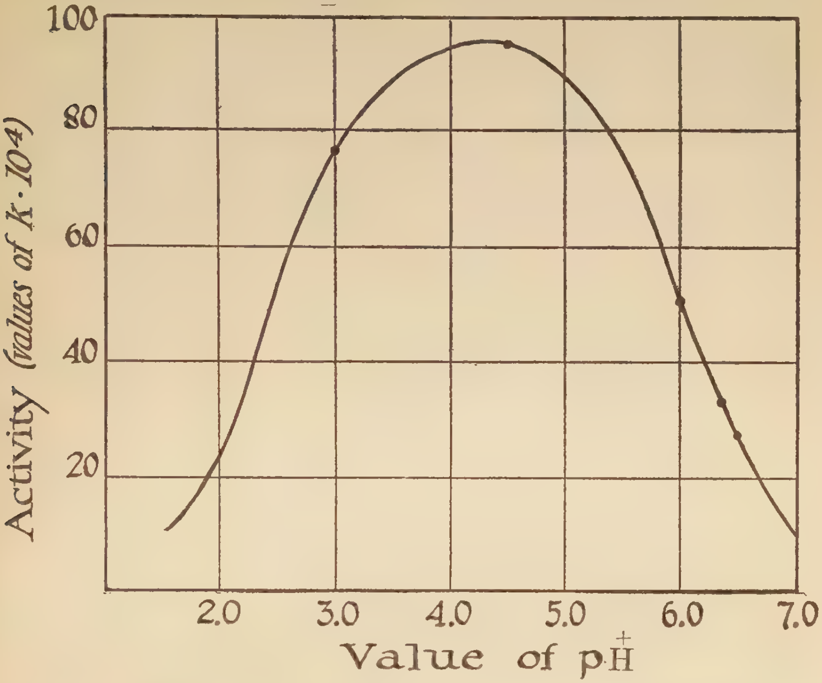

FIG. 7. INFLUENCE OF REACTION UPON THE ACTIVITY OF INVERTASE[418]

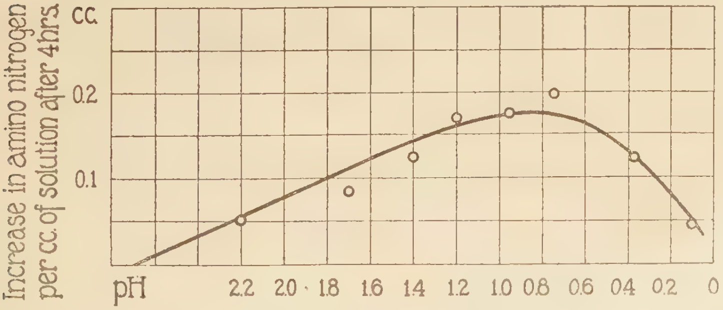

FIG. 8. INFLUENCE OF REACTION ON THE RATE OF DIGESTION OF EGG ALBUMIN BY PEPSIN[895]

of a compound between hydrochloric acid and pepsin, which has a greater proteolytic action than pepsin itself (Figure 8).

The length of time in which many enzymes remain potent is affected by the reaction of the medium in which they are kept. The optimal reaction for the stability of *Phaseolus* enzymes is at pH 6.5 to 7.0.[1147] Oxidase is very sensitive to acidity.[219] Although the optimum for trypsin is pH 9.5 and even higher, it is rapidly destroyed at pH 12 and is stable at pH 3.15. The influence of reaction on the filtration of enzymes and their thermostability has been previously discussed (see pp. 35, 39, 133).

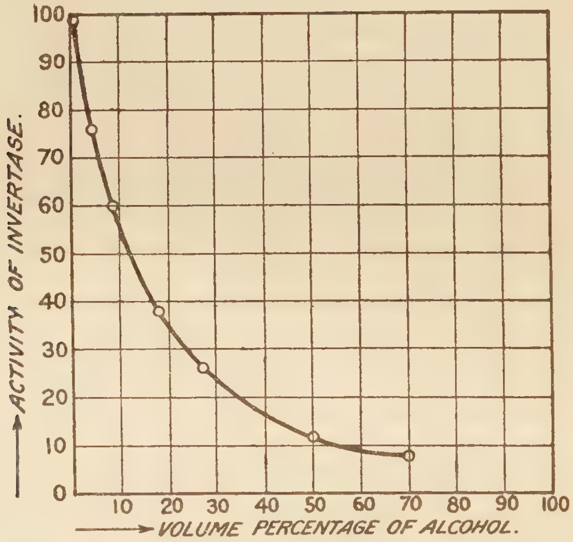

FIG. 9. INFLUENCE OF A PARALYZER UPON THE ACTION OF AN ENZYME[582]

6. ACCELERATORS AND PARALYZERS. *a. General accelerators and paralyzers.* The term accelerator has been suggested to designate a substance which has a stimulating action upon a catalyst.[646] A paralyzer is a substance which injures or inhibits the action of the catalyst. Certain paralyzers or inactivators, by their presence, paralyze the action of the enzyme. This inhibition is temporary and the activity of the enzyme is restored if the paralyzing substance is removed. Other paralyzers totally and permanently inactivate enzymes. The influence of chemical substances upon enzyme reactions may depend on their effect: (1) upon the enzyme itself,

stimulating or destroying it; (2) upon the substrate making it more or less readily available for the action of the enzyme; or (3) upon the reaction velocity of the enzyme. Ions other than hydrogen and hydroxyl are also important in regulating enzyme activity. Acids, bases and salts will, according to their concentration, either stimulate or injure enzymatic reactions by acting as positive or negative secondary catalyzers. Salts in concentrations less than 2 to 5 per cent usually have an accelerating effect upon enzyme activity and above this strength they are usually paralyzing, especially with certain enzymes such as catalase. It is possible that enzymes form salts with acids and bases which are either more or less active than the free enzyme itself. Ozone exerts a greater destructive effect than pure oxygen; the amount of destruction depends upon the concentration and activity of the enzyme solution.[1142] Certain enzymes (diastase, catalase) are sensitive to H_2O_2, while others (pepsin, trypsin) are not.[1242a] Catalase is particularly sensitive to oxidizing agents.

i. *Influence of the salts of heavy metals.* The injurious concentration of the salts of heavy metals depends upon the nature of the metal and the enzyme. The quantities of mercuric chloride necessary to stop completely the action of ptyalin have been accurately determined.[534] This inactivation is not permanent and the original activity of the enzyme can be restored by the addition of KHS. Trypsin and rennet are injured by small amounts of $HgCl_2$ but large quantities are needed to arrest its action completely. The addition of KCN restores the activity. The action of pepsin is but slightly reduced by $HgCl_2$. 0.005 mgm. of $HgCl_2$ per liter or a concentration of 1:200,000,000 is sufficient to reduce by 50 per cent the activity of many catalase preparations. A larger amount of $HgCl_2$—1:2,000,000—is required to reduce the activity of blood catalase 70 per cent.[1116]

The concentrations of the salts of the heavy metals required to injure the saccharifying action of diastase are as follows:[1796]

SALT	NORMAL CONCENTRATION REQUIRED
$AgNO_3$	0.00001
$AuCl_3$	0.00003
$HgCl_2$	0.000033
$CuCl_2$	0.00012
$NiCl_2$	0.0011
$CoCl_2$	0.01
$FeCl_3$	0.003

The effect of various concentrations of $HgCl_2$ upon a highly purified invertase preparation obtained from yeast is shown below:[401]

$HgCl_2$ IN 60 CC. OF ENZYME-SUBSTRATE MIXTURE	$K \times 10^4$ (REACTION CONSTANT)	RELATIVE VELOCITY OF THE ENZYME
mgm.		
0	735	100
0.03	739	100
0.06	575	78
0.15	289	39
0.30	148	20

The presence of buffers does not influence the effect of the mercury salt. An equilibrium is established between the enzyme and the $HgCl_2$ similar to that between a weak base and a weak acid.[401] One and one-half milligrams of $HgCl_2$ to 50 cc. of the enzyme-substrate mixture almost completely stops the action of invertase; it can be reactivated by saturating the solution with H_2S. The lower the concentration of the enzyme the smaller is the amount of mercury required to reduce its activity. The action of silver nitrate is similar but greater than that of the mercury salt.[401] The toxic effect of the salts of the heavy metals which results from the combination of the salt with the enzyme can be prevented by precipitating the metallic ions from solution. Enzymes inactivated by the salts of the heavy metals ($HgCl_2$, $AgNO_3$) may also be reactivated by sufficiently high concentrations of certain neutral salts of the alkalies and alkali earths.[655]

Gold chloride has a poisonous effect upon invertase similar to that of $HgCl_2$. Copper sulphate acts to a much less extent; cadmium and uranium salts have only a slightly poisonous effect. Further information on the action of metals upon enzymes may be obtained elsewhere.[102]

ii. *Influence of other salts.* Certain salts and ions greatly stimulate the activity of enzymes, as is illustrated by the influence of manganese salts on the action of oxidases. Other salts retard enzyme reactions, e.g., chlorides diminish oxidase activity at low temperatures. This fact is of practical value in the prevention of the browning of fruits and vegetables during their preparation for canning, preserving or drying.[1044] The neutral salts of strong monobasic acids accelerate the activity of salivary amylase while the neutral salts of weak acids reduce it.[265] Normal solutions of neutral salts are injurious to intestinal esterase, their toxicity increasing in the following order: chlorides, bromides, fluorides and iodides.[419] Arsenic salts and oxidizing agents, such as permanganate, are also injurious in certain concentrations. Some enzymes are more sensitive than others to sodium fluoride; it is injurious to pancreatic esterase in dilutions less than 1:80,000 and to stomach esterase in dilutions less than 1:16,000.[298] Liver amylase is inert in the absence of neutral salts. Monophosphates and aluminium sulfate, in dilute solutions, act very favorably upon enzymes but, in 2 per cent concentrations, they become distinctly injurious.[1098] Potassium bromate exerts a favorable influence upon the digestion of casein by trypsin in vitro, the effect being most marked with bromate concentrations of 1:100,000 to 1:200,000.[420a] Sodium chloride has practically no influence on the action of invertase at its optimal reaction (pH 4.5) but as the optimum is departed from in either direction there is an increasing salt effect.[418] Sodium chloride reduces the inhibitory action of calcium chloride upon lipase and a definite antagonism exists between the two in enzyme reactions.[421]

Much higher concentrations of the salts of alkalies and alkali earths are required for activating and injurious effects than of the salts of the heavy metals. Alkali salts of strong mineral acids usually become injurious at concentrations of 2.5 per cent. Some enzymes, such as diastase, are distinctly stimulated by the presence of alkali salts. The greatest effect is exerted by chlorides, less by nitrates and still less by sulfates, acetates and phosphates.[831] Most of the sulfates have an accelerating action; this is also true of dilute solutions of calcium and barium chlorides.[420] Larger quantities of sulfates as well as of $CaCl_2$ and $BaCl_2$ act injuriously. Numerous neutral salts, especially chlorides of alkalies and alkali earths, stimulate the hydrolytic action of pancreatic lipase, but not the synthetic processes. The action of these salts probably consists in saponifying the fatty acids, thus removing them from the system and thereby enabling the hydrolysis to proceed further.[961] The presence of salts not only influences the activity of enzymes but also their production by microörganisms.[1025]

iii. *Influence of organic substances.* Various organic substances exert a decidedly paralyzing effect upon enzymes and their removal leads to the reactivation of the enzyme. 0.000001 N hydrocyanic acid reduces the activity of catalase 50 per cent.[1116] This action is reversible and is independent of temperature. The proteolytic enzyme of yeast juice is weakened by 1 per cent hydrocyanic acid but not destroyed.[474] Papain is greatly stimulated by HCN but lipase, pepsin, rennet and invertase are scarcely affected by it.[467] Eight milligrams of HCN added to 60 cc. of an enzyme-substrate mixture reduces the activity of invertase only 10 per cent.[401]

Formaldehyde has a weakening effect upon enzymes, but the action is modified by the presence of proteins and their hydrolytic products.[363] The addition of 0.58 gram of formaldehyde to 60 cc. of a solution containing invertase produces 70 per cent inactivation (at 18°C.) in fifteen minutes and complete inactivation in eighteen hours;[401] 0.001 N formaldehyde reduces the action of catalase 20 per cent.[1116] In general, the inhibitory action of various organic substances, such as *p*-phenylenediamine, *p*-toluidine and formaldehyde, upon enzymes (invertase) depends upon the amount of free base. The effect of organic substances with a low acidity is greater than that of those with a high acidity.[408]

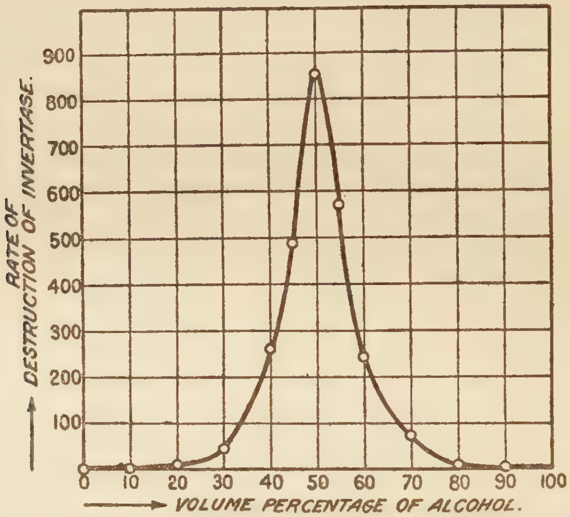

FIG. 10. INFLUENCE OF ALCOHOL UPON THE ACTIVITY OF INVERTASE[582]

Alcohols have an injurious effect upon enzymes (Fig. 9) by influencing the degree of dissociation and dispersion of the colloids; the enzymes are, however, destroyed slowly so that they are still active even if precipitated with alcohol and, if the contact has been comparatively short, the injury may be inappreciable. The dilution of the alcohol is an important factor in determining the degree of damage. The irreversibly destructive effect of alcohol on invertase (at 30°C.) begins with 20 per cent by volume, reaches a maximum at 50 per cent and falls, at 90 per cent, to the value of pure water[582] (Fig. 10). Undiluted alcohol is very injurious to some enzymes but others, such as emulsin and chlorophyllase, are still slightly active in the presence of 90 per cent alcohol.[582] Chloroform injures all enzymes to a varying extent[1204] while ether and acetone exert very little influence upon them.[987] Toluol is even less injurious. Organic substances also affect the solubility and surface tension of enzyme systems. Further information on the influence of organic substances and antiseptics upon enzymes may be obtained elsewhere.[351,404]

b. Specific accelerators and paralyzers. In additon to the general accelerators, there are those which are specific, as, for example, the various kinases, calcium salts for trypsin,[309] thrombin, pancreatic lipase, erepsin, pectase and rennet,[1038] phosphates for diastase and zymase, manganese and sometimes iron for certain oxidases. Asparagine is a specific activator for amylase,[360] and prussic acid for papain.[810] These specific activators, or co-enzymes have also been described elsewhere (see p. 19).

Some salts have a specific paralyzing action upon certain enzymes, i.e., sodium fluoride injures lipase to a greater extent than other antiseptics and it injures rennet but not pepsin and trypsin.[706] One of the examples of specific paralysis is the influence of the products of the reaction upon the action of the enzymes[43,727] and another is the action of anti-enzymes (see p. 19). Substances which adsorb enzymes or which combine with them are usually classified as anti-enzymes. These have nothing in common with specific antibodies, differing both in nature and in the mechanism of combination. There are also, however, true antibodies, which are specific for certain enzymes (see pp. 19, 20).[543]

7. LIGHT AND RADIATION. Light influences enzymatic reactions either by destroying the enzyme itself or by modifying its activity. Enzymes are far from being as sensitive to light as are toxins.[380] Injury by light usually occurs only in the presence of oxygen.[973,1319] The injurious effect of different rays of light upon peroxidase and blood catalase is in this order: white > blue > red > dark.[737,1319] The action of light on diastase and urease is dependent upon the dilution of the enzyme, upon the various impurities present and upon the

reaction. The greatest injurious effect occurs at the optimal reaction.[973] The addition of small amounts of fluorescent substances, such as eosin and chinilin red, to enzyme preparations increases the injurious effect of sunlight.[620,1190]

Ultraviolet rays also have an inactivating effect upon enzymes;[249,1092] the more purified the enzyme, the greater the destructive action of the rays.[973] The destruction of dry rennet by ultraviolet light is said to be a monomolecular reaction.[1190]

A very strong radium preparation exerts only a weakly injurious action upon rennet.[1092] Radium emanation increases the activity of certain enzymes such as diastase;[746] other enzymes are not influenced at all, while some are slightly weakened. Active or undissociated trypsin is decomposed by the beta radiations from radium emanation, and pepsin in solution is inactivated by the beta and gamma radiations.[591] Information on the influence of various radiations, including secondary rays and mesothorium upon enzymes may be obtained elsewhere.[288,404,591]

Röntgen radiation has no effect upon rennet, yeast, pepsin and pancreatin;[1019,1155,1233] the diastatic activity of urine, serum and various fluids after exposure to Röntgen rays for 20 minutes is unchanged.[712] However, slight but constant changes are produced in pepsin and amylase by X-rays.[1018] The amount of diastase in the blood of patients who have been exposed to Röntgen rays for either long or short periods is not altered although in some instances the blood sugar content is lowered.[311]

B. DISTRIBUTION OF ENZYMES

Enzymes are formed by all living cells, whether the latter carry on all the functions of an organism, as in the case of unicellular forms of life, or are devoted only to specialized functions as in higher plants and animals. The only difference between these two types of cells is that in the former all the enzymes are produced in a complex mixture, while in the latter the enzymes are usually specialized and are more readily separated, since different types of cells may produce certain enzymes in greater concentration than do other cells in the same organism. Some enzymes, such as diastase, are widely distributed throughout the animal and plant kingdoms, while others like laccase are found in but few plants.

CHAPTER V

ENZYMES OF THE HUMAN AND ANIMAL BODY

Life is just one enzyme reaction after another. It is popularly supposed that enzymatic activity in the human and animal body is limited to gastro-intestinal digestion but, in reality, it is difficult to name a body process in which enzymes do not play a more or less important rôle.

1. ENZYMES OF THE STOMACH. *a. Methods of studying gastric secretion.* The early experiments on digestion, such as those of Spallanzani[1159] in the eighteenth century, were performed by swallowing various kinds of food in linen bags or perforated wooden tubes which were examined after they had been passed in the stools. Human gastric juice was originally obtained by swallowing small sponges attached to strings, but this method was superceded by the stomach tube* which was introduced early in the nineteenth century by Physick.[970] However, the gastric juice collected by this method is so mixed with swallowed saliva and food that its study is not always accurate. The greatest advances have been made by those investigators who have worked with gastric juice obtained through gastric fistulae.[110,233] Beaumont,[110] by his observations on the secretion and functions of the gastric juice obtained from the wound of the stomach of Alexis St. Martin, laid the foundations for digestive physiology. Very little was added to the subject until Pavlov[953–955] published his experiments. He obtained very valuable information on gastric digestion in dogs by utilizing the uncontaminated gastric juice secreted in an artificially detached pouch of the stomach (Haidenhain operation). Carlson,[233] by his observations on the gastric juice obtained from an adult with a permanent gastric fistula, as well as by his subsequent studies, has greatly increased the knowledge of digestion. The present conception of gastric secretion has been recently reviewed.[233c,577]

b. Gastric secretion. There are two and sometimes three varieties of tubular glands in the mucosa of the stomach: the pyloric, fundic and cardiac, distributed as their names imply. The two latter are composed, in varying proportions, of two types of cells, the chief or central cells and the parietal or border cells. The former secrete the digestive enzymes of the stomach—pepsin, rennet and lipase—while the latter furnish hydrochloric acid. Gastric secretion is a continuous process, for the stomach is rarely empty, even in the morning before breakfast. Determinations made on patients with gastric fistulae have demonstrated that the stomach contents are increased during the summer months, probably as a result of changes in gastric motility and tonicity induced by heat. There is a continuous gastric secretion of 60 to 200 cc. daily in newly born infants before the ingestion of food. In guinea pigs gastric secretion occurs in utero.

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* The history of the stomach tube has recently been published.[203,567]

Gastric secretion consists of a series of cycles; first, that in response to the entrance of food into the stomach, or the digestive cycle, and then the long or short pause which has been designated the interdigestive period.[1012] The latter is associated with the phenomenon of hunger, as well as by a gastric secretion of low velocity. During the interdigestive period, there is an average of 50 cc. of residual secretion in the stomach of normal individuals.[454,1012] The acidity and peptic activity of this residual gastric material is lower than that of the digestive period. This loss of gastric acidity during the interdigestive phase has been explained as being due to a regurgitation of alkaline duodenal contents. Trypsin is said to be present in the gastric contents during this period.[1012] In the gastric contents of infants, however, trypsin is comparatively rarely found.[303] In men, during fasting, the flow of gastric secretion varies from 2 to 50 cc. per hour, but during the act of eating and throughout the period of gastric digestion, the rate may be increased to as much as 3.5 cc. per minute.[233] Gastric secretion is inhibited by external heat,[783,817,1312] fever[110,303] and other factors.[160,597]

There are at least three sources of stimuli which are responsible for the augmentation of gastric secretion: (1) the appetite; (2) the food in the stomach and, (3) the secretagogues or gastrin contained in the products of digestion. The rate of this appetite secretion varies directly with the palatability of the food and the degree of hunger and appetite.[233] Appetite secretion does not occur in newly born infants though it is present in puppies and in children over the age of three years. The gastrin theory[358] of gastric secretion has not been disproved, but much of the experimental work on which it is based is probably erroneous.[233c] Not only does the quantity of gastric juice vary with the ingestion of different kinds of food, but its quality is also apparently dependent upon the type of food. For example, the gastric juice secreted after a meal of bread has greater digestive powers than that after meat. Carlson, however, believes that it is the rate of secretion, rather than the type of food, which determines the quality of the gastric juice.[233]

c. *Enzymes of the gastric contents.* i. *Pepsin* was demonstrated in gastric juice by Eberle and Schwann in 1836.[1109] Beaumont, however, had previously described the digestion of protein by gastric juice.[110] Pepsin is present in the central cells of the gastric tubules as a zymogen or propepsin, but is quickly converted to active pepsin by hydrochloric acid. Apparently more pepsin is secreted after a meal of carbohydrate than after other foods. Commercial pepsin is made by extracting the ground up gastric mucosa of animals, usually pigs. The method of obtaining pepsin and of demonstrating its properties will be discussed later (see p. 202). The pyloric glands have an alkaline secretion containing a proteolytic enzyme which apparently is not a true gastric pepsin.[477]

Pepsin can be demonstrated in the gastric contents of infants immediately after birth;[710,720,1201,1211] its amount apparently increases during the first four months of life and the quantity is proportional to the infant's age rather than to his weight.[1049] Breast fed infants may secrete less pepsin than those who are artificially fed.[1049] In older children the maximal secretion occurs during the first thirty minutes after a carbohydrate meal and during the first forty minutes after the ingestion of milk alone.[40] The amount of pepsin in the gastric contents of infants suffering from digestive disturbances is usually reduced but increases during improvement.[811,838,1001] In cases of pylorospasm it is higher than normal.

In both adults and children the amounts of hydrochloric acid and pepsin secreted are not always parallel,[741,1001] but in the gastric contents of patients suffering from hypoacidity the secretion of pepsin is low and of those with hyperacidity it is high.[566] The enzymes of the stomach may be a more correct indicator of its condition than the HCl content.[149,793]

ii. *Gastric rennet* (*lab*). The presence of a milk coagulating substance in the stomach has long been recognized. Like pepsin, it originates in the central cells of the gastric tubules as a zymogen, prorennin or prochymosin, which is activated after secretion by HCl. Rennet is present in the gastric juice of fish so a milk diet is apparently not essential for the secretion of this enzyme. Because rennet is practically always found wherever proteolytic enzymes are present, there has been some doubt of its being a separate enzyme (see p. 197). The question has not as yet been conclusively answered, but the preponderance of the evidence at present is in favor of rennet being a distinct enzyme. Its properties are described elsewhere (see pp. 197–200). There is a marked parallelism between the amounts of pepsin and rennet in the gastric contents of infants.[237,811] These enzymes are present from birth,[510,720,1148] but their amounts apparently increase during the first few weeks of life.[1006,1211]

iii. *Gastric lipase.* The fact that lipase is so readily destroyed by the acidity of the gastric juice and that fats fed to experimental animals are usually recovered unchanged from duodenal fistulae has led to the erroneous belief that the stomach does not secrete lipase. However gastric juice or a glycerol extract of the gastric mucosa can liberate fatty acids from emulsified fat, egg yolk and cow's milk.[1231] Furthermore, pure gastric juice obtained from dogs, on which the Pavlov operation has been performed in order to prevent any possibility of contamination with duodenal secretion, is markedly lipolytic if the dogs have been fasting or if the gastric acidity is low.[585] The lipase of very acid gastric juice is inactive, while that of specimens which contains acid in moderate amounts is only active after the acid has been neutralized or reduced by the introduction of peptone into the stomach.[585]

Takata[1187] has recently published an extensive study of the gastric juice obtained from dogs which had a "Pavlov's small stomach." He determined the lipolytic activity by titrating the amount of fatty acids liberated from an emulsion of milk fat globules (see p. 141) and found the optimal reaction to be pH 4.9 and that the lipase of a dog's stomach is active from pH 2.5 to 8.0. Even when the dogs' stomachs are empty, a viscid mucous material, neutral in reaction, of marked lipolytic activity is secreted at the average rate of 1 cc. per hour. After a meal of raw meat, the amount and acidity of the gastric juice rise rapidly and reach a maximum in thirty to forty minutes. The lipolytic activity diminishes as the acidity rises and is usually not demonstrable an hour after the meal. Ten hours after the meal the acidity falls and there is a distinct lipolytic action. The secretion of gastric lipase is apparently not repressed by the secretion of acid but the enzyme is probably destroyed by the acid, for if the stomach is washed out and the washings immediately neutralized, lipase can be demonstrated at any time throughout the entire course of gastric secretion after a meal, even during the period of maximal acidity. Takata has also demonstrated that lipase can be precipitated from gastric juice by shaking it with finely pulverized magnesium oxide and centrifuging. If the precipitate is washed with water, recentrifuged and then dissolved in HCl (avoiding an excess of acid), a solution is obtained which is powerfully lipolytic and practically protein free. Lipase can be precipitated from this solution by the addition of alcohol or acetone. This precipitate when dried in vacuo retains its lipolytic activity indefinitely. He also found that even though active gastric juice is immediately neutralized, it loses its lipolytic activity within six hours at room temperature. However, it remains active for several days, or even weeks, when kept in a refrigerator. The addition of 2 cc. of 0.2 N HCl to 1 cc. of gastric juice or lipase solution destroys its activity within fifteen to forty minutes. The activity of a lipase solution is still present, though reduced, after five hours' contact with an equal amount of 0.04 N HCl. The addition of a small amount of egg-white retards the destructive action of HCl, but larger amounts inhibit the action of the lipase. Starch does not affect the influence of HCl on lipase. Four-tenths per cent Na_2CO_3 destroys the lipolytic action of lipase in an hour. The addition of protein does not check this destruction.

Monobutyrin and tributyrin are readily hydrolyzed by the gastric juice of dogs while the amyl ester of valeric acid, ethyl butyrate, olein, triacetin and an emulsion of palmitin and stearin are split to a much less extent. Squalene, an unsaturated hydrocarbon obtained from shark liver oil,[1206] is not affected by gastric juice, although it is readily decomposed by pancreatic juice. Emulsification is not necessary for lipase action; unemulsified olein, olive oil, fats of milk and egg yolk are hydrolyzed. The gastric lipase of dogs does not hydrolyze pure lecithin, although the pancreatic juice is strongly active. Lecithin has no effect upon the activity of lipase, although it influences pepsin.[698] It does, however, increase the stability of milk fat emulsion used as a substrate. Gastric juice loses much of its lipolytic activity when the mucin particles are removed, when the proteins are precipitated by heating, or when mixed with adsorbents, such as animal charcoal.[1187] Gastric lipase can be differentiated from pancreatic and intestinal lipase by the extreme sensitiveness of gastric lipase to alkali. The first of these enzymes is found in the stomachs of fasting individuals, while the two latter are frequently present in the stomach, especially after the administration of atropin, which opens the pylorus.[1187]

Lipase is present in the stomachs of infants as early as the second week of life and in the stomach of rabbits immediately after birth.[1113] The whey of woman's milk increases the action of gastric lipase in infants.[484]

iv. *Amylase.* Gastric contents, as obtained by stomach tube, frequently contain swallowed saliva and regurgitated duodenal contents, in addition to the true gastric secretion. By determining the amounts of amylase and trypsin present, one can ascertain whether the gastric contents are contaminated with saliva or duodenal contents, for pure gastric secretion probably does not contain either of these enzymes. Saliva possesses a very active amylase and it is usually assumed that trypsin is absent, while both enzymes are present in the duodenal contents. If the specimen of gastric contents contains amylase alone, it may be presumed to be contaminated with saliva, and if both amylase and trypsin are found, the specimen probably contains duodenal contents with or without saliva. Specimens of gastric contents which have been demonstrated to be free from saliva and duodenal material are the only ones upon which reliable conclusions in regard to gastric secretion can be based, yet this factor is usually disregarded in discussions of gastric analyses. Less than one-third of forty-nine specimens of gastric contents of normal infants which were examined were free from saliva or duodenal contents.[303] The presence of saliva in gastric contents is not remarkable, for infants as well as well bred adults swallow almost all of the saliva secreted. Specimens containing saliva are not visibly different from those free from saliva, so that amylase tests are necessary for their differentiation. Because of the presence of bile, contamination with duodenal contents can usually be detected so that trypsin tests are probably not essential.

v. *Catalase.* The amount of catalase in the gastric contents of normal individuals is very small but is increased in cases of gastritis and carcinoma.[894]

d. Gastric digestion. Although various distinct gastric enzymes probably exist, gastric digestion is practically limited to the proteolytic activity of pepsin and rennet. Of a given amount of protein in the stomach of an infant 48 per cent passes through the pylorus as peptones or proteoses, 20 per cent is undigested and 20 to 30 per cent may be absorbed from the stomach.[1200] However, in animal experiments little or no protein is absorbed in the stomach under normal conditions and the products of gastric digestion of proteins are largely proteoses.[749] Some native proteins, such as a serum-albumin, are not acted upon by trypsin unless they have previously been partially digested by pepsin and HCl. The action of the stomach is really only a preliminary stage in the process of digestion. The food is thoroughly mixed and forced into the duodenum in small amounts with the proteins digested to the proteose stage. In fact, human surgery, as well as animal experiments, has demonstrated that the stomach is not essential to digestion and nutrition. There have recently been published a comprehensive review of the literature on gastric digestion in infants[258] and a history of gastric secretion and gastric digestion from the time of Galen to the contributions of Pavlov.[742]

2. ENZYMES OF THE DUODENAL CONTENTS. The pancreas and duodenum, like the stomach, have a continuous secretion, the human pancreas secreting 7 to 8 cc. per hour and the total daily output amounting to nearly 900 cc.[757] At the height of digestion this organ can produce in one hour a quantity of pancreatic juice equivalent to its own weight. A normal infant can secrete from 5 to 25 cc. of duodenal material per hour.[303] The duodenal contents, in addition to bile, contain the enzymes, amylase, invertase, lipase, rennet and trypsin.[387] Most of these enzymes are said to be secreted by the pancreas as zymogens and are activated by the enterokinase of the succus entericus, or secretion of the cells lining the duodenum and small intestine. This latter secretion also contains erepsin.[1220]

a. The duodenal enzymes of adults. Various diets affect the character of the duodenal secretion (obtained by duodenal tube); in normal individuals the lipolytic activity is always, while proteolytic and amylolytic activities are almost always, greater after the ingestion of olive oil than after an ingestion of protein or carbohydrate food. Water is a less potent stimulant of external pancreatic secretion than are food substances. After the appearance of food substances in the duodenum there is a latent period before the flow of enzymes and bile begins. The time coincidence of the onset of the flow of bile and of pancreatic juice suggests that they both have a common stimulant. The duodenal contents of fasting adults contain enzymes as well as bile pigment.[793] In a patient with a duodenal fistula, the ingestion of a mixture of protein, fat and carbohydrate stimulated the duodenal secretion of protease and lipase; the eating of fat alone produced a secretion of amylase and protease, and ingestion of protein and a very small amount of fat caused a marked production of all three of these enzymes.[155, 1144]

The hydrogen ion concentration of the human duodenum has been determined by inserting an electrode through a fistula; the most alkaline value is pH 8.23 and the most acid pH 5.9 with an average of pH 7.02.[587] Differences in the type of food ingested apparently do not greatly affect the reaction of the duodenal contents although they may become more acid after the ingestion of proteins and slightly alkaline after a meal of fat and carbohydrate foods.[793] Very acid foods, such as buttermilk, will of course increase the acidity of the duodenum.[303]

In acid specimens of duodenal contents the activity of the duodenal enzymes is often reduced and, therefore, clinical conclusions should only be drawn from those determinations which are made on neutral or alkaline specimens. In gall bladder disease the duodenal lipase is reduced, although the amylase and protease activity may be practically normal.[1143] The average amount of each enzyme in the duodenal juice of fasting patients suffering from gall bladder disease may be less than 50 per cent of the normal content and in a considerable number of patients, one or more enzymes are absent.[972] In chronic pancreatic disease, sprue and fatty diarrhoea, the various duodenal enzymes are also decreased.[1143]

Abnormalities are usually found in the enzymatic activities of the duodenal contents of patients suffering from some organic lesion involving the pancreas primarily or secondarily and in those in whom clinical, operative or autopsy data indicate the possibility of a derangement of the external secretory function of the pancreas. Chronic pancreatitis, acute pancreatic necrosis, cancer of the head of the pancreas, acute and chronic cholecystitis, infectious (catarrhal) jaundice and lesions obstructing the pancreatic duct are accompanied by marked abnormalities in the enzymatic activity of the duodenal contents. In achylia gastrica and pernicious anemia, no abnormalities in duodenal secretion may be demonstrable. The presence or absence of bile does not apparently affect enzymatic activity.[793] In diabetes mellitus, the external secretory activity of the pancreas, especially the lipolytic and proteolytic enzyme secretion, is greatly altered.[627] However, experiments on dogs with pancreatic fistulae have apparently demonstrated that different mechanisms are involved in the external and internal secretions of the pancreas, for insulin does not augment the external pancreatic secretion, while a profuse secretion follows the injection of secretin.[705]

Pyloric juice contains enzymes similar to those which are found in the stomach but pepsin and lipase are much more concentrated in the latter. Pancreatic steapsin can break up the lecithin molecule;[1187] pancreatic lipase is resistant to cocaine but is affected by high concentrations of strychnine.[197] Many clinical tests have been devised for the determination of the enzymatic activity of the duodenal contents.[89,90,214,374,793,1143,1161,1162]

b. The duodenal enzymes of infants. If iron pills coated with salol are fed to infants and, with a catheter in situ, the urine is examined every five minutes, the excretion of iron can be demonstrated within forty to sixty minutes in children from one to three years of age; within fifty-eight minutes, in those three to five years old and, within seventy-three minutes, in children from six to ten years of age. In normal infants the excretion continues from twenty-four to twenty-six hours, while in those who are ill it lasts longer occasionally up to one hundred and forty-four hours. These data probably illustrate the rate of duodenal digestion, for it is more likely that the salol coating is digested in the intestine than in the stomach.[967] In infants suffering from atrophy, the "exudative diathesis" or rickets the amount of duodenal lipase is reduced. In patients who do not gain in weight when fed woman's milk, even though suffering from no obvious disease, duodenal lipase is absent. Age, time of feeding and the character of the diet do not produce appreciable variations in the amount of duodenal lipase.[497] There is apparently no differ-

TABLE X

Duodenal contents of normal and diarrhoeal infants[303]

STATE OF DIGESTION	NUMBER OF SPECIMENS	pH	UNITS OF AMYLASE (DIASTASE)	UNITS OF TRYPSIN	PERCENT OF SPECIMENS CONTAINING A HEAVY GROWTH OF B. COLI
					per cent
Normal	23 (duodenal tube)	6.2	17.1	74.5	0
Convalescent	16 (15 duodenal tube, 1 autopsy)	6.0	19.5	30.7	0
Diarrhoea	6 (autopsy)	5.3	0	3.2	83
Dysentery	5 (autopsy)	5.1	11.6	10.3	0

ence in the ability of the duodenal juice of healthy breast fed or artificially fed infants to digest the protein of woman's or cow's milk.[460]

In digestive disturbances in infants, especially in diarrhoea and to a lesser extent in bacillary dysentery, the activity of the duodenal amylase and trypsin is decreased and the hydrogen ion concentration of the duodenal contents and the numbers of colon bacilli in the duodenum are increased (Table X). While, of course, no definite conclusions can be drawn until more observations are collected, the suggestion has been made that five factors may be responsible for the production of diarrhoea in infants: (1) a primary cause which is still undetermined but which may be different in individual cases, i.e., external heat, fever, infections, overfeeding with an improper diet, etc.; (2) a reduction in the activity of the duodenal enzymes and secretions as a result of the primary insult; (3) an accumulation of undigested and unabsorbed food material in the duodenum as a consequence of the reduction in enzymatic activity; (4) the invasion of this food material by organisms of the colon group from the caecum and large intestine; and (5) the fermentation of this food material by these organisms and the formation of irritating end-products which accelerate peristalsis and produce diarrhoea.[303] Additional evidence for this theory is presented on page 71.

The starch digestion of infants is frequently impaired in intestinal disturbances and in systemic diseases; the presence of undigested starch in the stools may precede an intestinal disease by several days.[1145] These observations would seem to corroborate the belief that a diminished secretion of intestinal enzymes is one of the factors which may be responsible for the production of diarrhoea in infants. That a failure of the external pancreatic secretion may be a factor in chronic intestinal indigestion in infants (coeliac or Herter's disease) is also suggested by necropsies on two of these cases in which extreme fatty infiltration of the liver and pancreas and atrophy of the pancreatic acini were found.[257,788] These changes, of course, may have been secondary to the patients' inanition—similar to those observed in adults dying of war edema. Unchanged protein passes through the intestinal wall into the blood stream in infants suffering from malnutrition.[34] Whether this phenomenon indicates an increased permeability of the intestinal walls in this condition, or an absence of intestinal enzymes which permit the ingested protein to remain undigested, is still to be proven. In infants suffering from undernutrition which has resulted from a diet insufficient in quantity and not from a gastro-intestinal disturbance, there is no evidence of defective absorption or abnormal utilization of food.[474]

c. The duodenal enzymes of animals. In experimental animals in which the biliary duct is obstructed the presence of trypsin in the bile is apparently capable of producing gangrenous cholecystitis, even in the absence of infection.[1099] It has been suggested that the toxic features of ileus may be due to the cleavage products of proteins which result from tryptic action, for dogs can be immunized against the toxins of ileus by raising the antitryptic titer by injections of trypsin.[1099]

Additional evidence that a lack of duodenal enzymes may perhaps be responsible for diarrhoea (see p. 70) is the fact that depancreatized dogs even though treated with insulin die with symptoms of diarrhoea two to seven months after their pancreases have been removed. These dogs cannot be fed polysaccharrides without developing diarrhoea and there is evidence of failure of fat and protein absorption.[24,241,445] However, if they are fed raw pancreas they remain alive indefinitely and do not have diarrhoeal symptoms. The removal of sufficient pancreas to render animals diabetic results in a much greater accumulation in and a slower removal of fat from the blood plasma than if a normal amount of pancreas is present.[145]

In dogs, after a protracted secretion of pancreatic juice has been maintained at a constant rate by the continuous injection of secretin, the concentration of lipase, trypsin and amylase diminishes at a similar rate.[36] These results are contrary to an earlier report that the concentration of lipase falls more rapidly than that of the other two enzymes.[703] The lipase of the pyloric juice of dogs is far weaker than that secreted in a "Pavlov small stomach."[1187] The pancreatic digestion of pigeons has also been studied.[708]

3. ENZYMES OF THE SALIVA. The most important constituents of saliva are mucin, urea, inorganic salts and the enzymes ptyalin (diastase) and maltase. In dogs and some other animals, ptyalin is normally absent from fresh saliva. Saliva is secreted by three glands, the parotid, sublingual and submaxillary. The main difference in the secretion of these three glands lies in the fact that the parotid forms no mucin and in man produces more ptyalin than the other two glands. *Ptyalin* is a typical diastase whose optimal reaction is pH 6.1 to 6.2; the optimal temperature is 46°C.[415] Saliva obtained from the parotid ducts, or extracts of the salivary glands of

horses, does not digest starch. This inactive saliva, or zymogen, is activated in the mouth by orokinase, which is secreted by the buccal and possibly the lingual glands. Orokinase is also present in human saliva. When tested with cooked starch the amylolytic action of the mixed mouth saliva of horses is equal to that of human saliva, and, with raw starch, is greater than that of human saliva.[946]

It is often stated that the saliva of newly born infants cannot digest starch. However, diastase can be demonstrated in the saliva of all infants, even in those who were born 1½ months prematurely, by inserting cotton plugs in their mouths and then extracting and examining the contents.[261, 891] The amount of diastase increases with the age of the infant and at one year is identical with that of adults. The saliva of strong, vigorous infants contains more diastase than that of those who are weak. The presence of starch in the diet of infants apparently makes no difference in the amount of diastase in their saliva.

In cachetic patients, the amount of salivary diastase is less than that in normal subjects; in those suffering from stomach affections the salivary diastase is low if hypochlorhydria is present, but higher than normal if the patients have hyperchlorhydria or a gastric ulcer.[232] Undigested starch in the stomach apparently delays peptic digestion and accelerates that of rennet, but if the starch has previously been digested by saliva, this effect is destroyed. Saliva alone, probably because of its thick mucin, delays the clotting of milk in the stomach and causes the production of a finer curd.[865]

4. ENZYMES OF THE FAECES. The lipolytic action of faeces is probably due, partly at least, to the presence of bacteria.[844] The difficulty of distinguishing between the enzyme activity of the intestinal organisms and of intestinal secretions greatly detracts from the value of enzyme studies of the faeces. Catalase has been demonstrated in faeces.[894] The evidence in regard to the relation of digestive disturbances and stool enzymes is contradictory: according to one report[507] there is no difference between the amount of ferments in the stools of infants suffering from diarrhoea and in those of normal children, while acording to another[718] no trypsin or, at the most, only very faint traces can be demonstrated in the filtrates of stools of infants suffering from digestive disturbances. The amount of trypsin in the stools of rachitic patients is said to be high.[759a] The quantity of diastase in the faeces of normal and undernourished infants varies widely but is apparently increased in patients suffering from rickets.

In undernourished children and in those suffering from alimentary toxicosis, the amount of lipase in the stools is usually normal but in rachitic patients it may be increased.[568]

5. Enzymes of the Blood. Many studies have been made of the enzyme content of the blood serum, leucocytes, lymphocytes, red blood cells and blood platelets. Various methods have been used and it is to the differences and often the fallacies in these methods that the present confusion in regard to blood enzymes is largely due. Not only have many purely laboratory experiments been made, but a considerable literature has accumulated in regard to the clinical interpretation of blood enzyme determinations.

a. Enzymes of blood serum. i. *Serum diastase.* Since Magendie's report in 1846[775] that blood could split starch into dextrin and glucose, and that it, therefore, contained a diastatic enzyme, a mass of conflicting data has accumulated, certain investigators doubting the presence of this enzyme in the blood and others endeavoring to interpret physiologically or clinically the variations which they have found. Probably these discrepancies are due to the different methods used for the determination of diastase.

By the viscometric method[304] no starch liquifying activity can be demonstrated in the serum of normal individuals. However, the addition of human serum to preparations of *Asp. oryzae* greatly increases their starch-liquefying activity as compared with controls without serum. Serum also increases the activity of duodenal diastase, but inhibits the tryptic activity of pancreatic extracts and of duodenal contents. Heating the serum to 58°C. reduces its power to accelerate diastatic activity and to inhibit tryptic digestion. It has been suggested that serum may contain a diastase accelerator as well as antitrypsin.[907] By other methods, in which saccharogenic activity is measured, considerable amounts of diastase have been found in the blood of normal individuals; in the very young the quantities are usually lower. There is usually no relation between the diastatic activity of the blood and the food eaten by the individual.[262, 726, 1253] However, according to one report[311] the blood diastase is increased during dietary hyperglycemia and it decreases during the dietary treatment of hyperglycemia, whether or not the individual has diabetes. The blood of normal individuals may have a normal amount of sugar, with normal or low diastatic activity; or a high sugar content with increased or low diastatic activity, but never a low sugar content with high diastatic activity.[311] The level of blood diastase is usually constant throughout the day whether or not the patient is suffering from diabetes.[525]

It is generally believed that there is an increased breaking down of glycogen to dextrose in diabetes; this may be due to the accumulation of some substance in the body, which stimulates the glycogenolytic activity of the cells, for the blood diastase is not augmented to a sufficient extent to account for this change.[839] The diastatic activity of the blood is not increased in phlorhizin glycosuria and piqûre causes no change in the diastatic activity of the blood, but in both of these conditions there is a tremendous mobilization of sugar. There is probably no relation whatever between the blood diastase and the degree of glycogenolysis and glycosuria for many patients have marked glycosuria with a low blood diastase[83,726] and in some cases of severe diabetes there is an almost complete absence of diastase from the blood,[80] particularly if the patient also has syphilis.[863] From a study of 55 diabetic patients and a large number of normal adults in whom the amounts of diastase in the blood and urine were estimated simultaneously it was concluded that the presence of this enzyme was more or less accidental and of little or no value in the diagnosis, prognosis, or treatment of diabetes mellitus.[195,525]

However, the statement is sometimes made that usually in diabetes the higher the level of the blood diastase, the more severe is the diabetes, and that the internal secretion of the pancreas inhibits the diastatic activity of the blood.[311,863] In dogs, hyperglycemia produced by adrenalin and morphine and hypoglycemia due to insulin are associated with a fall in blood diastase and a rise in liver diastase and it has been suggested[262] that the function of the blood diastase is its assistance in the utilization of glycogen and that it is not a waste end-product of metabolism on its way to excretion by the kidney, as is usually believed.[233,664,936]

The large amounts of diastase sometimes found in the blood of patients suffering from severe diabetes instead of being due to any particular change in the rate of production of the ferment by the diabetic individual may possibly have been caused by retention of the diastase by a damaged kidney, for chronic nephritis is quite frequent in patients who have had diabetes for a long time.[726] In patients whose kidneys are impaired, the amount of diastase in the blood may be high and that of the urine low.[525] There is, however, a marked variation in the amount of diastase in the blood of patients suffering from nephritis, and often no apparent relation between the diastatic activity and the type of the disease or the progress of the lesion, so determining the amount of blood diastase in cases of nephritis is not a reliable method of measuring renal functon.[726,1164]

The diastase content of blood serum (and urine) increases steadily during pregnancy. Colostrum is rich in enzymes, which, like those of the blood and urine, decrease in amount after the child is born; the source of the surplus diastase is probably the placenta.[1217] There is nothing characteristic in either the blood diastase or blood sugar of patients suffering from various stages of cancer.[195,311] The amount of diastase in the serum of normal and undernourished infants varies within wide limits.[568] The administration of various amylolytic enzymes does not change the diastatic action of the serum; they are apparently excreted unchanged in the urine, for the amount of urinary diastase is increased.[787]

ii. *Serum lipase.* The serum of rabbits and guinea pigs has a very active lipase (monobutyrinase) while that of swine and cattle serum is weak.[1032] Extraction with ether destroys this lipolytic activity of blood serum.[29] Pancreatic lipase and serum lipase of rabbits differ in several essential characteristics: the optimal reaction for the former is between pH 8.3 and 9.0, while that of the latter is pH 8.0.[1035] The optimum for serum lipase is also different from that of gastric lipase. Calcium, barium, magnesium and manganese salts increase the activity of pancreatic lipase, but have no influence upon serum lipase. The inhibiting effect of NaF is much less upon pancreatic lipase than upon serum lipase. Pancreatic lipase is effective on both heterogenous and homogenous systems, while the activity of serum lipase is confined to a homogenous system. These observations would seem to indicate that serum lipase does not represent pancreatic lipase which is being carried by the blood as a waste product to be excreted by the kidneys as has been maintained in regard to serum diastase.

The amount of lipase in the serum of healthy children varies widely.[539] The lipase content of the serum of undernourished children is apparently normal, of those suffering from alimentary toxicosis it may be normal or decreased and that of rachitic patients it is increased.[133,568] The amounts of lipase and complement in sera are often parallel. Human serum in some instances inhibits the activity of pancreatic lipase.[197]

One of the protective mechanisms of the body against the tubercle bacillus may be the lipolytic activity of the serum. In favorable cases of tuberculosis the lipase content of the serum is high, while in patients whose progress is downward it is low.[225,837] Lipase digests the fatty envelope of the tubercle bacillus, and antigens made in this way have been used with apparent benefit in the treatment of tuberculosis in animals.[39]

The serum lipase of patients suffering from jaundice of hepatic origin is resistant to quinine, while that of patients suffering from other conditions, as well as that of normal individuals, is destroyed by this drug.[1037] It has been suggested that this difference in the effect of quinine upon serum lipase is due to the fact that the lipase in the serum of patients suffering from hepatic jaundice is of liver origin. This phenomenon may be useful as a method for distinguishing hepatic from hemolytic jaundice in doubtful cases;[1037] the determination of the amount of lipase in the serum may also be an aid in the diagnosis of pancreatitis.[649] According to this method of differentiation of the lipases in the serum, the lipase in the serum of patients suffering from cirrhosis of the liver or from tuberculosis is of liver origin while that of the serum in cases of pernicious anemia or in younger diabetic patients is not. The blood of the older diabetic patients contains liver lipase.[1216] The fact that serum lipase is inactivated by quinine while liver and nerve lipase are quinine resistent has been used to demonstrate the influence of psychoses upon the disintegration of the organs and the penetration of the organ lipases into the blood.[205]

iii. *Serum protease.* Blood serum treated with chloroform causes proteolysis of gelatin and casein; ox serum, especially its globulin fraction, contains a weak proteolytic enzyme which is active at an alkaline reaction.[542] There is a proteolytic enzyme, which requires a neutral or alkaline medium, in the serum of the tuberculous pleural exudate of a dog, after it has been treated with 0.2 per cent acetic acid.[920] After severe chloroform poisoning, the sera of dogs acquire the ability to digest protein at an acid reaction.[921] Although protease is present in the sera of guinea pigs, rabbits, cats, oxen and dogs, there is little or none in normal human serum unless the antiprotease is removed.[617,907] It is possible that there is a relation between the amount of pepsin in the stomach and that in the blood and urine.[941] An increase in the amount of serum ereptase (peptidase, erepsin) invariably precedes or accompanies the crisis or lysis of lobar pneumonia, but in fatal cases, the amount of serum ereptase is usually less than that of normal individuals.[964] This enzyme may be concerned with the dissolution of pneumonic consolidation.

Antipepsin. It has been suggested that a disproportion between pepsin and antipepsin is the predisposing factor in the development of gastric ulcers.[651,652,675] This theory is supported by the report that antipepsin can be found in the blood of normal individuals but none in that of patients suffering from ulcers of the stomach.[375] However, the results of the examinations of the blood of fifty patients who had been operated upon, do not sustain this hypothesis so the question is still open.[924]

iv. *Serum anti-trypsin.* Since the first description of the anti-tryptic action of the blood serum in 1893[562] many observations on anti-enzymes have been made and numerous interpretations advanced. The anti-tryptic power of the blood of animals is apparently increased after injections of an extract of dried leucocytes,[618] of trypsin,[18,124] of an emulsion of tissue cells,[184] of kaolin, proteases, peptone, *B. welchii*[1307] and other bacteria.[617] It is increased in patients, as well as in animals, after the administration of thyroid glands.[816] Profound intoxication with chloroform or phosphorus apparently increases the amount of proteolytic enzyme of the blood and, in some instances, causes the complete disappearance of the anti-enzyme. Smaller repeated doses of chloroform or phosphorus produce a progressive increase of anti-enzyme.[921] The sera of guinea pigs, rabbits and horses lose their anti-tryptic action after treatment with kaolin, starch, agar, or bacteria. The serum of dogs inhibits trypsin and leucoprotease, which act in an alkaline medium, but has no effect upon serum protease, which is active at an acid reaction.[617] The parenteral introduction of yeast enzymes, liver diastase and pancreatic lipase into animals does not produce an inhibiting effect in the serum.[13] In view of the multiplicity of measures which will apparently increase or decrease the anti-enzyme activity of the blood, this substance cannot be regarded as a true antibody, specific for its own antigen.

There is no uniformity of belief in regard to the nature of the substance responsible for the anti-tryptic action of serum; it has been attributed to lipoids which can be removed by extraction with ether,[1110] to the presence of compounds of the unsaturated fatty acids,[617] to serum proteins[1192] and to the albumin fraction of the serum.[707] Although from a comparison of the anti-tryptic activity of serum albumin and charcoal it might seem that the equilibrium between trypsin and anti-trypsin is governed by adsorptive phenomena,[534] the reaction between charcoal and trypsin is probably not analogous to the reaction between anti-trypsin and trypsin, or to that between solid protein and either trypsin or pepsin.[898] The anti-tryptic action of the blood is presumably due to the formation of an inactive but dissociable compound by the inhibitive agent and trypsin.[590] The conditions of equilibrium of this reaction are apparently governed by the law of mass action. The behavior of this equilibrium is similar to that between various inhibitive agents and enzymes.

It is possible that the anti-tryptic content of the blood is related to sensibility to anaphylactic shock. If a suspension of fresh liver is introduced into the peritoneal cavities of guinea pigs, the anti-tryptic content of the blood is nearly trebled as a result of the autolysis of this liver tissue.[659] If these animals are sensitized to horse serum, shock cannot be produced with the same dose of horse serum which kills control guinea pigs (which have had a liver suspension, previously boiled to prevent autolysis, introduced into their peritoneal cavities before sensitization). There is no change in the serum protease after the first injection of foreign protein, but if acute anaphylactic shock is produced, there is an instantaneous increase in non-specific protease and a decrease in anti-enzyme.[617] Several investigators have reported in sensitized animals the presence of enzymes specific for the protein used as antigen.

The variations in the anti-enzymatic activity of the blood in many conditions have been studied, but the results obtained have been very contradictory. There is a diminution of anti-enzyme in the blood of patients suffering from general and local pyogenic infections.[1271] Nevertheless variations in the anti-enzyme content of the blood, particularly in chronic disease, may have no relation to leucocytosis. The amount of anti-trypsin is increased in the blood of most patients who are suffering from carcinoma, sarcoma and tuberculosis, accompanied by wasting and cachexia.[191] This increase has been designated "the cachexia reaction." However, a similar reaction has been reported in exophthalmic goitre unassociated with wasting and anemia.[815] The anti-enzymatic activity of the serum is said to be increased in severe anemia, chlorosis[188] and pregnancy.[490,1195] At the site of an inflammatory reaction the anti-enzyme of the surrounding fluid usually limits the action of the leucoprotease of the leucocytes to the intracellular digestion of engulfed solid particles and prevents the proteolysis of the tissues.[922] However, if the inflammatory reaction is very intense, the amount of free leucoprotease liberated by disintegrated leucocytes may be sufficient to overcome completely the anti-enzyme in the exuded serum, and tissue destruction results. One of the factors in bacterial intoxication may be a disturbance of the enzyme-anti-enzyme balance in the serum which results in the proteolysis of serum protein, with a subsequent liberation of toxic split products.[617]

Although the anti-tryptic titer is increased in cases of iodide poisoning, the administration of small doses of potassium iodide may diminish the anti-tryptic activity of the blood serum and hasten the softening of caseous material.[617] A similar explanation has been suggested for the absorption of syphilitic gummata which follows the administration of potassium iodide. However, it has not been demonstrated that enzymes, such as leucoprotease, which are inhibited by the anti-enzyme of the blood serum, are concerned in the absorption of tubercles or gummata.[922]

The anti-tryptic power of the blood serum of patients suffering from pneumonia increases during the earliest stage of the disease, decreases during the crisis, increases again immediately after the crisis and then subsequently returns to normal.[53,143] During the crisis, in addition to the anti-enzyme, the non-coagulable nitrogen and the proteases of the serum are decreased and the amount of non-specific protease increased.[617] Apparently the anti-enzyme content of the blood in cases of myelogenous leucemia is not altered,[618] although in fatal cases there may be a reduction before death and the serum may cause proteolysis.[1271]

v. *Serum autolysis.* The addition of ethyl ether, petroleum ether, benzine, toluol, cholesterol, lecithin, triolein and tripalmatin does not influence autolysis, but if treated with chloroform, acetone, methyl, ethyl and isobutyl alcohol, the serum undergoes proteolysis. The original non-autolytic state does not return after the chemical activators are removed by vacuum dialysis or extraction with ether or petroleum ether.[1310]

vi. *Blood catalase.* The catalase of blood has been studied extensively. It is found chiefly in the erythrocytes; oxalate plasma, as well as lymphocytes, polynuclear cells and blood platelets contain very little catalase. The catalase content of the blood and tissues of different animals differs, as shown in Table XI. The catalase content of the blood is more or less constant in healthy individuals.

During the development of the embryo, the catalase content of the blood and certain organs, which are relatively poor in catalase, remains more or less constant, while that of the liver and nerve cells progressively increases. It has been assumed that the presence of catalase determines the rate of oxidation which occurs at different stages in the life-cycle of an organism.[222] However, this hypothesis is probably incorrect.[850] A rapidly growing cancer of the liver contains less catalase than normal liver.[147] This decreased catalase content is also true for other tumors,[1050] as well as for the blood and organs of men suffering from tumors.[1293a] The term catalase index, which has been introduced, refers to the number of grams of H_2O_2 liberated from 30 cc. of 1 per cent H_2O_2 by 1 cc. of a 1:1000 blood solution, divided by the number of millions of red blood cells in this 1 cc. of diluted blood.[1174] Normal blood has a catalase index of 4.6; blood of a patient suffering from secondary anemia, 4.8; polycythemia, 3.2; pernicious anemia, 8.5. However, there is some doubt as to the value of this index.[850] Catalase formation goes hand in hand with peroxide production. This has been shown to hold true of the catalase content of the blood at high altitudes.[1222]

TABLE XI

Catalase content of the blood and tissues of various animals[93]

ANIMAL	LIVER	BLOOD	NERVES	LUNGS	HEART	MUSCLE
Man	700	920	240			55
Cat	1,390	540	180	250	32	21
Rabbit	370	460	390	98	65	16
Horse	5,600	390	900	200		90
Frog	2,960	140	440	100		26
Dog	624	51	210	52	15	9
Pigeon	1,480	14	340			

The figures given in this table have been obtained by multiplying the number of cubic centimeter of O_2 produced in 20 cc. of 1 per cent H_2O_2, in ten minutes at 18°C., by the ratio of $\frac{0.10 \text{ grams of tissue}}{\text{grams of tissue actually used}}$.

vii. *Blood oxidase and peroxidase.* Altitude up to 7000 feet does not affect the oxidase power of the blood of men, cattle, rabbits and guinea pigs.[599a] No peroxidases have been demonstrated in the serum of horses, cattle, sheep, and other animals.[196]

viii. *Blood glycolase.* The sugar content of blood kept at room temperature progressively diminishes and in the course of two to four days may entirely disappear as a result of glycolysis. Glucose added to the sample is similarly destroyed. This process, which often renders sugar determinations worthless, may be prevented by a method recently devised.[1045]

ix. *Bilirubin enzyme of the plasma.* The assumption is frequently made that an extracellular enzyme exists in the body which has the power to convert hemoglobin into bile pigment, but attempts to demonstrate an enzyme of this nature in the plasma or in the spleen of dogs have so far been unsuccessful.[1017]

b. Enzymes of the polynuclear leucocytes. i. *Leucoprotease.* A glycerin extract of purulent sputum or fresh sputum from an acute abscess digests fibrin or coagulated protein in a weakly alkaline medium; the thin fluid from a tuberculous "cold" abscess has no such power.[858] These observations in 1888 were probably the first descriptions of leucoprotease or the proteolytic enzyme of pus cells. Even earlier than this peptone had been observed in pus and in the urine of patients suffering from purulent diseases, such as empyema, peritonitis and meningitis.[368,569,777] A complete review of this subjects, parts of which are abstracted in this book, has recently been published.[922]

The methods used for the demonstration of leucoprotease have been based upon the liquefaction of gelatin, the digestion of casein, fibrin, coagulated egg albumin, plates of coagulated serum, and the determination of the amount of nitrogen in non-coagulable substances before and after digestion. Leucoprotease may be extracted from pus cells by glycerin,[858] by treatment with absolute alcohol and ether,[920] and by other methods. Fresh leucocytes do not dissolve fibrin but, if they are killed by being heated at 55°C., their enzymes are set free and digestion occurs.[859] A purified enzyme may be prepared from leucocytes by allowing them to autolyze and then treating the autolysate with alcohol and ether, filtering through a porcelain filter, again adding alcohol and ether and then drying the white precipitate in vacuo over sulfuric acid.[618] The final product is yellowish brown and somewhat hygroscopic. It digests blood serum, gelatin and casein. In solution, it is destroyed by temperatures between 70° and 75°C., but, in dry form, it is active after being heated from 70° to 95°C. and is finally destroyed at 100°C. This enzyme is especially resistant to formalin, being active even though dissolved in a 10 per cent solution of this antiseptic. In addition to being present in leucocytes, leucoprotease has been found in monkey, dog and rabbit organs in which leucocytes are numerous, such as bone marrow and spleen.[920] It is formed within the leucocytes before they leave the bone marrow. Proteolysis can not be demonstrated in the spleen and bone marrow of foxes, cats, various rodents, pigs, sheep, oxen and horses[618] nor is leucoprotease found in the leucocytes, spleen and bone marrow of hens.[920] It has been suggested that leucoprotease and the α-protease of the spleen[545] may be similar, as the optimal reaction for both is slightly alkaline.

The evidence in regard to the relation of leucoprotease and trypsin is conflicting. The injection of animals with one or the other of these enzymes increases the inhibiting action of the serum for both.[618] On the other hand, it has been observed that normal human and turtle serum inhibit trypsin, but the latter has little or no effect on leucoprotease.[618,1271] The end-products of the digestion of Witte's peptone by leucoprotease and trypsin are similar,[618] while those resulting from the digestion of casein by these two enzymes are quite different.[617] Leucoprotease, unlike trypsin, does not destroy diphtheria toxin;[619] it does not hemolyze red blood cells. The effect of leucoprotease on bacteria will be discussed elsewhere (see p. 84). It has been suggested that complement is derived from the polynuclear leucocytes and that it has the characters of a proteolytic enzyme.[813] However, complement is not identical with leucoprotease, nor with the protease of macrophages, for these enzymes resist temperatures which destroy complement.

The sputum and lung exudate obtained at autopsy from patients dying during the later stages of lobar pneumonia, contains a proteolytic enzyme, while during the earlier stages of the disease proteolysis is prevented by the anti-enzyme of the serum of the exudate.[753] The proteolytic enzymes contained in the cells obtained from pneumonic lungs is active between pH 6.7 and 7.3, but is inactive at more acid reactions. There is also a peptolytic enzyme in pneumonic exudates which is active between pH 4.8 and 8.0 and whose optimal reaction is between pH 5.2 and 6.3. One explanation for pneumonic resolution is that, as the disease progresses, the acidity of the pneumonic exudate increases, thus inhibiting the proteolytic enzyme but activating the peptolytic ferment.[753]

The fluid from a tuberculous abscess, unlike the pus of acute inflammation, does not contain a proteolytic enzyme.[859] However, if the contents of a "cold abscess" contain polynuclear leucocytes, proteolysis can be demonstrated. The blood of patients suffering from myelogenous leucemia contains a very active proteolytic ferment (possibly due to a reduction of anti-enzyme, see p. 79), while that of patients suffering from lymphatic leucemia does not. The enzyme is in the basophile cells and transitional forms as well as in the granular myelocytes.[1322] The normal spinal fluid, unlike other body fluids, contains neither leucoprotease nor anti-enzyme, but in meningitis due to pneumococci or streptococci, the leucoprotease liberated by disintegrated leucocytes gives the spinal fluid proteolytic activity which may cause irritation and increase the severity of the disease. A study of the enzymes of the spinal fluid in pathological conditions has recently been published.[348]

ii. *Anti-leucoprotease.* Blood serum inhibits the proteolytic activity of leucoprotease;[920] heating serum to 75°C. for one-half hour destroys this inhibiting substance, but a temperature of 65°C. has no affect. There is no anti-leucoprotease in cerebro-spinal fluid, bile, milk or normal urine but albuminous urine inhibits leucoprotease.[859] The leucoprotease of the dog is inhibited by the serum of man, ox, dog, cat, goat and rabbit, but not by the serum of pigeons and hens.[920] Anti-leucoprotease can be precipitated by the complete saturation of blood serum with $(NH_4)_2SO_4$, but not by one-third or half saturation. A given amount of serum can inhibit only a limited quantity of leucoprotease, for example, 2.5 cc. of serum may completely inhibit 20 mgm. of dried leucocytes, but fail to prevent proteolysis if a larger quantity of enzyme is employed. The variations in anti-leucoprotease in the serum of patients with various diseases has been discussed elsewhere (see p. 78).

iii. *Fibrin protease.* Fibrin dissolves when placed in neutral or weakly acid or alkaline solutions. The effect of different temperatures and the formation of protein disintegration products have demonstrated that enzymes are concerned in the process. This digestion of fibrin is probably caused by leucocytes enmeshed in the fibrin, for pure fibrin free from leucocytes is almost insoluble in normal saline, unless leucocytes are added.[1064] A proteolytic enzyme which is active in acid, neutral or alkaline media can be obtained from blood fibrin.[86] In the fibrin of inflammatory exudates, obtained by injecting turpentine into the pleural cavity of a dog, two enzymes can be demonstrated,—leucoprotease, which is abundant during the early stage of the inflammation and disappears after the second day, and a second enzyme which is abundant after the second day. The former is active in an alkaline medium and the latter at an acid or neutral reaction.

iv. *Erepsin of leucocytes.* In addition to leucoprotease, erepsin has been obtained from leucocytes.[617,950]

v. *Bacteriolytic substance of leucocytes.* An extract of the leucocytes of normal animals is bacteriolytic.[473] This phenomenon is probably comparable to the observation that although the proteolytic enzyme of leucocytes has no bactericidal action for *B. typhosus, staphylococci* and *streptococci,* it can digest dead typhoid and colon bacilli; living bacilli resist digestion.[619] Leucoprotease can digest pneumococci.[617]

vi. *Oxidase and peroxidase in leucocytes.* The fact that leucocytes of marrow origin contain granules which can be stained blue with solutions of benzidine or copper sulfate—the so-called oxidase or peroxidase reaction—is frequently used to differentiate these cells from those of lymphatic origin. These granules are present in myeloblasts but not in lymphoblasts. They are also said to be absent in chicken leucocytes.

c. *Enzymes of the mononuclear cells, lymphoprotease.* In contrast to the leucoprotease of polymorphonuclear leucocytes, which has its optimal activity in an alkaline medium, the proteolytic en zyme of macrophages or large mononuclear cells (lymphoprotease) is most active at an acid reaction.[920] Lymphoprotease is more sensitive to heat than leucoprotease, being impaired by temperatures between 60° and 70°C. and destroyed at higher temperatures. It closely resembles the autolytic enzyme of parenchymatous tissues. In acute lymphatic leucemia, however, it has been reported that the amount of proteolytic enzyme in the lymphocytes is similar to that of polynuclear leucocytes.[859] Apparently the large lymphocytes in cases of acute lymphatic leucemia contain a proteolytic enzyme which is active in an alkaline medium, while the small lymphocytes in the chronic form of the disease do not,[752] although it is sometimes stated that there is no difference in the enzymatic activity of these two types of cells.[384]

d. *Enzymes of the red blood cells.* The protease which is said to be contained in red blood cells[1084] is probably leucoprotease, for the red cells of rabbits, dogs, horses and men cannot hydrolyze protein unless leucocytes or tissue extracts are added.[975] The lipase of the erythrocytes is resistant to cocaine and strychnine.[197] The catalytic action of the blood is due chiefly to the catalase content of the erythrocytes (see p. 79).

e. Enzymes of the blood platelets. Although the statement is made that blood platelets contain an active peptolytic enzyme,[5] proteolytic or lipolytic enzymes cannot be demonstrated in the blood platelets of rabbits and dogs.[1054] Very little is known of the other enzymes of platelets.

6. Enzymes of the Urine. *a. Urinary diastase (amylase).* In healthy individuals, by methods which measure saccharogenic activity, diastase can be demonstrated in the blood serum, urine and practically all other body fluids. The level is usually constant in the serum, but in the urine it is subject to diurnal variations, due partly to digestive functions.[1168] The total day's diastase secretion should be considered, rather than the number of units in any one specimen,[1148] for the concentration of urinary diastase is often inversely proportional to the amount of urine voided.[1164] The total daily urinary excretion of diastase is fairly constant in normal individuals, an average of from 10 to 20 units (Wohlgemuth[1295]); in adults it is usually unaffected by diet, reaction of the urine, the presence of bacteria or any other abnormal urinary constituent except blood. In infants fed exclusively with milk, the amount of urinary diastase is small, but it increases as soon as the diet contains starch.[278] In normal infants as well as in those who are undernourished there are considerable fluctuations in the amount of urinary diastase.[568] The diastatic activity of the urine is increased by the administration of various amylolytic enzymes.[787]

The optimal reaction for the activity of urinary diastase, with a modified Wohlgemuth method[1298] and a phosphate buffer, is pH 6.1; more uniform results probably can be obtained if all specimens of urine are brought to this hydrogen ion concentration before the diastase content is determined. Diastase clings to urinary deposits and, therefore, all specimens should be thoroughly shaken before the diastatic estimation is made.[333] The activity of the enzyme does not appreciably diminish in urine kept for many weeks, provided toluol is present.[278] No anti-amylase has been demonstrated in urine.[1168]

The determination of the amount of urinary diastase has been recommended as a test of renal function,[1295,1298] but the results are unreliable.[1058] In many cases of nephritis, the urinary excretion of diastase is diminished and there is an accumulation of diastase in the blood,[278,1148,1168,1295,1298] especially if a large proportion of the kidney is damaged.[1164] However, there is no constant relationship between the extent of the renal lesion and the concentration of diastase in the blood or urine.[1148,1164] In eclampsia, the urinary excretion of diastase is usually high and the concentration in the urine may be higher than that in the blood.[278,1247]

In patients suffering from some but not all types of pancreatic lesions there may be an increase in the urinary excretion of diastase,[218,1148,1168, 1295,1298] and the level of the blood diastase may also be elevated.[1168] The determination of the amount of diastase in the urine after attacks of suspected pancreatitis may be of some diagnostic value.[649] In one patient suffering from an abscess of the pancreas perforating into the stomach, the urinary diastase rose markedly just before the perforation occurred and in two cases of gall stone colic the diastase excretion suddenly increased.[46] The urine of patients who are suffering from rickets has a high diastatic power.[33,333,568,1248a] In strictly treated diabetic patients the amount of urinary diastase is often low.[278,453]

b. Urinary pepsin. Pepsin has been demonstrated in human urine; its amount is often, though not always, parallel to that of the blood and stomach.[741] In infants suffering from nephritis, the quantity of pepsin and erepsin in the urine is appreciably increased.[568]

c. Urinary lipase. The amount of urinary lipase excreted by undernourished children and those suffering from alimentary toxicosis is within the normal range but it is increased in rachitic patients.[568] In glomerulonephritis and in catarrhal icterus, the lipase content of the urine is markedly increased.[1067]

d. Urinary catalase. The amount of catalase in the urine is an index of the number of cells therein and the urine of patients suffering from pyelitis can be differentiated from that of normal individuals by its catalase content.[894]

7. ENZYMES OF THE TISSUES AND ORGANS. *a. Autolytic enzymes.* Animal organs kept at 37°C. with antiseptics undergo changes more or less similar to those occurring during the digestion of proteins in the alimentary canal.[1073] This process was first called "autodigestion" but later the term "autolysis" was introduced.[602] Sodium carbonate checks autolytic processes[1112] and hydrochloric acid increases the rate of autolysis of the liver[142] and of practically all organs except the pancreas.[541]

The autolysis of the pancreas and liver of dogs and pigs proceeds farthest in solutions whose reaction is between pH 5.0 and 6.0, i.e., an optimum between that for tissue pepsin and trypsin.[316] Apparently autolysis rather than being due to a specific enzyme is caused by tissue pepsin and trypsin, as it is most rapid and complete when both of these enzymes can work simultaneously. If a piece of tissue is first treated with weak acids and subsequently transferred into an alkaline solution, the autolysis proceeds much farther than if the tissue has been from the start in an alkaline solution. This fact is probably due to the effect of the tissue pepsin in an acid medium and the subsequent activity of the tissue trypsin at an alkaline reaction. In view of these and similar observations most of the autolytic reactions can be explained without the assumption of hypothetical agents, such as "proenzymes," activators," "anti-enzymes" and a primary protease[315] (see below).

The proteins of the spleen autolyze only at a neutral or acid reaction and not in an alkaline solution; rapid hydrolysis occurs at pH 5.68.[181] An acid medium (pH 4.5) is optimal for the autolysis of beef kidney, beef liver, pig liver and other tissues.[182] In the normal living cell with an average reaction of pH 7.4, the tissue proteins are in the form of base protein combinations which are not substrates for the cellular autolytic enzymes, and they remain undigested as long as the normal hydrogen ion level prevails. When acid develops, acid protein salts are formed which are available for primary cleavage catalyzed by an enzyme designated as the *primary protease* of the tissues (β-protease[545] or endotryptase[508]). This primary cleavage results in end-products of the general character of proteoses and peptones, which are then rapidly converted into amino acids by the active ereptase present in the tissue. Although it is conceivable that autolysis is not catalyzed by specific enzymes (see above), there is evidence that this primary protease is quite different from trypsin and pepsin and also that an ereptic type of enzyme is present in tissues.[182] The autolysis of pancreatic tissue, probably because of its trypsin content, usually proceeds best at reactions which are more alkaline than those optimal for other tissues.[182]

Caseous material from tuberculous lesions or from the lungs of patients who die of tuberculous pneumonia does not undergo the active autolysis often observed with lobar pneumonia,[859] and an anti-tryptic agent can be extracted from the caseous material of tuberculous lymph nodes and lungs which may be responsible for this failure of caseous material to undergo liquefication.[617]

Atrophic changes in the muscles and other tissues of the body, according to one theory, may be caused by chemical liquefaction of the tissue proteins, catalyzed by enzymes which are present in the cells.[182] Atrophy, or "in vivo autolysis," apparently proceeds to a much further degree than autolysis outside of the body for the living muscle may, after section of its motor nerves, lose 50 per cent or more of its protein mass, while in the same period in vitro, at the most favorable hydrogen ion concentration, the autolysis of an excised muscle seldom exceeds 15 per cent.[246] In prolonged excessive exercise and fatigue, sufficient acidity may develop in a muscle so that some of the contractile protein fraction is lost by autolysis.[246] This is accompanied by a disproportionately large loss of strength and may be a factor in determining the length of time required for complete recovery after excessive exercise.

b. Proteolytic enzymes of the tissues. Enzymes exist in the spleen which are capable of hydrolyzing fibrin and peptone.[316,854] In the liver, spleen, pancreas, leucocytes and gastric mucous membrane of recently killed dogs and pigs there are pepsin-like enzymes, which split native proteins to peptones but not further, and for whose action the optimal hydrogen ion concentration is about pH 3.5.[316] In addition, all of these tissues contain proteolytic enzymes of the type of trypsin or erepsin which attack only peptones or peptides and split them into amino acids. The optimal hydrogen ion concentration for these latter enzymes is between pH 7.6 and 8.0 and their activity is checked in slightly acid solutions.[316] In some of the tissues, one or the other of the enzymes predominate, as for example, the pepsin in the stomach and the trypsin in the pancreas. Unlike the less specialized enzymes, pancreatic trypsin and gastric pepsin can easily be extracted from the cells.

The spleens of oxen, horses and pigs apparently contain at least three different proteolytic enzymes: (1) α-protease which digests spleen substance and casein at pH 8.8; (2) β-protease which hydrolyzes the same substrates at pH 5.4, and (3) erepsin, which is not active against casein, but which exerts its most powerful action on Witte's peptone at pH 7.5 to 8.5.[545] These three enzymes can be separated by successively precipitating and extracting minced spleen with acetic acid, casein solution, and NaCl. The first filtrate contains all three enzymes, the second β-protease and erepsin and the third α-protease. If fresh spleens are kept at reactions more alkaline than pH 6.5, the enzymes lose much of their activity but this process can be prevented by keeping the reaction at pH 5.2. Aqueous extracts of spleens obtained at pH 5.2 contain varying amounts of all of the three enzymes mentioned above. Brain tissue contains erepsin acting at pH 7.8 and a proteolytic enzyme active at pH 4.3 to 5.6.[545] An erepsin-like enzyme active at pH 5.6 and a protease whose optimum is pH 7.6 are present in the thymus.[1268] Proteases have also been demonstrated in the placenta,[774] tissues and liquids of the eye,[236] muscles[1055] and in the white and yolk of eggs.[673]

There is a proteolytic enzyme, active in a weakly acid medium, in tuberculous tissue;[920] this enzyme is similar to that of the large mononuclear phagocytes and its presence in tuberculous tissue is probably due to these cells in the tubercles. Leucoprotease, which requires an alkaline medium, is only present when the tuberculous tissue is first formed and contains leucocytes. The bone marrow and spleen of patients suffering from myeloid leucemia contain a very active proteolytic enzyme; lymph nodes contain this enzyme in proportion to the myeloid transformation they have undergone. The bone marrow of normal individuals contains an active proteolytic enzyme, the spleen a weak one and lymph nodes none. The amount of this enzyme in the organs of patients suffering from lymphoid leucemia is similar to that of normal individuals.[618] The optimal reaction for the protease obtained from malignant human and rat tumors is pH 7.0; this enzyme is six to seven times as active as the protease obtained from normal rat tissue.[424]

c. *Tissue catalase.* Different parts of the same tissue may have a different catalase content.[850] A rapidly growing cancer of the liver contains less catalase than normal liver.[147,1050,1293]

d. *Tissue diastase.* Diastase is found in the cells of the pancreas, salivary glands, liver, placenta, eggs, muscles, kidney, tissues, and liquids of the eye as well as in blood and lymph.[135,161,236,509,673,774] Most of the tissue diastase is probably formed by the pancreas and distributed throughout the body by the blood stream.[1087] However, although there is a sharp drop in the blood diastase immediately after the pancreas has been completely removed, the amount returns to normal levels within four or five days. This observation would seem to indicate that although the pancreas may be the principal source of diastase under normal conditions, other tissues of the body can produce this enzyme.[726]

e. *Tissue glucosase.* The addition of fresh muscle tissue and insulin to ordinary β-glucose results in its conversion to γ-glucose.[762]

The addition of insulin alone or of insulin plus blood has no such effect. It would seem that if the blood-sugar of normal individuals is in the γ-form, the β-glucose absorbed in the intestinal tract is not converted into the γ-form in the blood but that this conversion is an extravascular process. The muscle tissue is inert after it has been excised two hours, even when kept at 37°C., so presumably its active principle is closely connected with vital processes. The conversion of β-glucose into γ-glucose is reversible but the reversed process is very slow; it is not hastened by the addition of normal gastric juice.

f. Tissue glycolase. Tumor tissue in vitro, both aerobically and anaerobically, has marked glycolytic power;[1250] the same process apparently occurs in the living animal.[279] Glycolysis in the tissue of transplantable rat tumor is much more active than in liver tissue.[841]

g. Tissue lipase. Lipases and esterases have been demonstrated in extracts of various organs and tissues, such as pancreas, liver, eye, spleen and mucous membrane of the intestines. The lipase of the kidneys and liver is resistant to cocaine and strychnine.[197] Organ extracts, except that of the pancreas, if made with ether, have no lipolytic activity.[29] It has been reported that an enzyme, which hydrolyzes monophosphoric esters, is present in bones and cartilage in which active ossification is occurring and that it cannot be found in any cartilage prior to the appearance of the center of ossification.[785]

h. Tissue oxidases. In various neurological conditions, the process of degeneration is associated with a diminution of the oxidizing enzymes of nerve cells.[781] An oxidase has also been demonstrated in the eye[236] and in eggs.[673]

i. Tissue reductases. Practically all of the tissues except necrotic portions of carcinomata have a reducing power which varies according to the type of tissue; kidney, liver and testis have the highest reductase content while plasma and serum are devoid of it.[1230]

j. Bacteriolytic substance of the tissues. Extracts of the small intestine and liver of guinea pigs will dissolve typhoid bacilli. This lytic principle can be transmitted by passage through successive typhoid cultures.[699]

k. Tissue desaminase and desamidase. Amino acids injected into the blood stream of normal individuals and of those suffering from catarrhal jaundice disappear within ten minutes.[1047] Apparently this fact can be utilized in detecting impairment of the deaminizing function of the liver, for in patients suffering from yellow atrophic hepatic neoplasms or from cirrhosis, the injected amino acids persist in the blood longer than 10 minutes. A desamidase has been demonstrated in placental tissues.[774]

l. Insulin. That insulin, the extract of the islands of Langerhans of the pancreas, will increase the ability of a diabetic patient to digest sugar is undoubted. The exact mechanism by which this result is accomplished is still unsolved, although several interesting hypotheses have been advanced. Insulin itself probably contains but few of the properties of the enzyme secreting portion of the pancreas. By viscometric methods, practically no trypsin and only a slight trace of amylase can be demonstrated in insulin. Without being an enzyme itself, insulin apparently stimulates the pancreas and other organs and tissues to greatly increased enzymatic activity.

Insulin does not alter the rate of the fermentation of glucose by yeast,[1203] it has no influence on the fermentation of glucose by *B. coli*[797] and does not affect the rotation properties of glucose.[762] These observations would seem to indicate that the action of insulin in increasing the ability of a diabetic patient to digest sugar is not exerted upon the glucose molecule itself.

It is possible that insulin, in reducing the amount of sugar in the blood of diabetic patients, may cause the sugar to form some as yet unknown intermediate storage product.[192] This substance may perhaps be a compound of sugar and phosphate, for the injection of insulin causes a decrease in the concentration of inorganic phosphate and potassium, as well as of glucose, in the blood of normal dogs.[192] The reversible process (glucose$\rightleftarrows$lactic acid) plays a considerable part in carbohydrate metabolism and, as in diabetes the ability to form lactic acid from glucose is lacking, the action of insulin may be to restore this defect and so to remove the symptoms of diabetes. Studies of the respiratory exchange would seem to indicate that neither in normal nor diabetic individuals can the hypoglycemia, which results from the injection of insulin, be explained by an increased carbohydrate oxidation.[165]

8. ENZYMES OF THE SKIN. Enzymes have been found in human skin which has been freed from blood. Its diastase is more active than that of liver tissue and almost as strong as that of serum. The lipase, which is also present in subcutaneous tissue and in fat from

the kidney region and mesentery, is resistant to quinine and atoxyl. A peptolytic enzyme, an active catalase and a phenolase, which oxidizes pyrocatechin better than epinephrin, can also be demonstrated. In addition, trypsin, neucleotidase and autolytic enzymes are present in the skin.[1299]

9. Enzymes of Milk. Lipase has been found in colostrum and in woman's milk at all times of the day, at the beginning and at the end of the lactation period.[112,134,484] This enzyme is not present in the milk of cows, goats, rabbits and dogs. The suggestion has been made that the lipase of woman's milk may explain its superiority in the feeding of infants who are suffering from nutritional disturbances. The diastase of milk is largely in the butter fat, therefore milk with a high fat content contains a larger amount of diastase. Colostrum is much richer in diastase than ordinary milk. The milk of animals fed on green and mixed feeds contains more diastase than that of those fed on dry feed. Milk diastase is weak in its starch liquefying action, but it readily digests soluble starch; 100 cc. of milk being capable of dextrinizing 0.05 to 0.10 grams of soluble starch in sixty minutes at 30°C. Sodium chloride and blood serum have a stimulating action upon milk diastase. Milk does not contain any invertase.[253a] The bactericidal action of cows' milk, which is often attributed to the leucocytes contained, may possibly depend upon the oxidizing enzymes of milk.[518] This action is destroyed by heating to 115°C., a precaution sometimes necessary in cheese factories in order that the activity of the lactic ferments used as starters may not be inhibited.[792]

10. Protective Enzymes (Abderhalden's Abwehrfermente). Abderhalden[10] demonstrated in the serum of pregnant women a proteolytic enzyme which he believed was specific for placental protein. It is certainly plausible that such an enzyme should develop in the serum of pregnant women to protect the body from the absorption of placental protein. He later reported that the serum of patients suffering from cancer contained a proteolytic enzyme which was specific for the protein of cancer tissue. This conception of specific protective enzymes was soon transferred from the experimental laboratory to the hospitals and as a diagnostic procedure was studied by many investigators.[10]

There are various theories to explain the mechanism of this reaction. It is possible that, under the conditions of Abderhalden's method, the restraining influence of the anti-enzyme is removed so that the normal serum protease becomes active.[617] According to another hypothesis,[198] the Abderhalden reaction consists of two stages (1) a combination of the substrate (antigen) with the antibody and (2) the adsorption of the serum antitrypsin by this combination and the consequent liberation of the normal proteolytic ferments of the blood which then digest the protein of the serum.

With a modification[764] of Abderhalden's method, it is said that not only can pregnancy be diagnosed as early as the second month but, in nearly all instances, the sex of the foetus can be correctly predicted.[1094,1115] However, at present it is usually agreed that the Abderhalden reaction is worthless as a diagnostic measure and that the serum of men often digests placental protein quite as well as the serum of pregnant women.[87,455,1117,1302]

11. Enzymes as Antigens. It has been suggested that the substances related to proteins and associated with proteolytic enzymes, as well as the toxins of bacteria, are concerned with the production of the fever which accompanies suppurative processes.[619] The temperatures of rabbits usually rise one hour after subcutaneous or intravenous injections of leucoprotease or of pancreatin, whether or not the enzymes are active or have been inactivated by heat. After the injection of trypsin and proteases into dogs, the amount of serum protease is increased and the antitryptic titer usually also rises.[617] The amount of enzyme bears no relation to variations in the temperature or in the number of leucocytes of these animals. Pepsin administered by mouth has no effect upon the blood pressure or cardiac rate, but if injected subcutaneously the blood pressure usually falls, although the cardiac rate is unchanged.[741] Thirty or forty minutes after the subcutaneous injection of pepsin, there is radiographic evidence of an increase in the peristalsis and muscular tone of the large intestine.[741] The toxic symptoms produced by burns and anaphylactic shock are comparable to the poisoning produced in animals by the injection of trypsin.[968] Preliminary adsorption by charcoal removes the toxic effect of trypsin, and trypsin poisoning in animals can be prevented by injections of India ink. The effect of enzymes as antigens has also been discussed in connection with serum anti-trypsin (see p. 77).

12. Unknown Enzymes. It is occasionally necessary to search for a new enzyme. For instance, in an effort to demonstrate the

mechanism by which hemoglobin is transformed into bilirubin, extracts of the blood, spleen, and other organs were examined for the presence of an enzyme capable of producing this change.[1017] In this search for an enzyme, different hydrogen ion concentrations, different dilutions of substrate and different temperatures of incubation are necessary, because an enzyme may be active under certain conditions and completely inactive under others and, therefore, a wide range of conditions must be employed.

CHAPTER VI

PLANT ENZYMES

1. DISTRIBUTION OF ENZYMES IN PLANTS. Enzymes are unequally distributed in various parts of dormant and growing plants as well as in dormant and germinating seeds. In some instances, as, for example, emulsin and amygdalin in the seeds of bitter almonds, the enzymes and substrate are present in different cells of the same tissue, so that, on crushing the tissues, the enzymes are brought into contact with the substrate. In other instances, the enzymes are formed in the cells containing the substrate. The enzymes most commonly found in plants are diastase, protease, maltase, oxidase and catalase. Pectase, invertase, rennet, lipase, cytase, peroxidase, reductase, emulsin, myrosin, phytase, inulase etc., occur less frequently. Some of these enzymes are present in one part of a plant and some in others, e.g., the leaves of *Beta vulgaris* contain invertase, diastase and maltase, the stem possesses invertase, diastase, inulase and emulsin, while the roots have the last three enzymes but no invertase.[1026] Tobacco plants contain invertase, diastase, reductase and emulsin in the leaves and seed at all stages; lipase, inulase and protease are present in small quantities; the oxidase content diminishes at the end of the ripening process.[918] The amount of enzyme is usually greater in plants that are rich in the specific substrate, for example, diastase is usually more abundant in leaves that are rich in starch, than in those which are high in sugar, and more plentiful in leaves which are exposed to light, and therefore contain more starch than shaded leaves.[376] The presence of certain minerals also affects the enzyme content of plants; barley grown in sand containing phosphate has 2.3 times as much diastase as the plants grown in the absence of phosphate.[391]

Diastase is found not only in the great majority of seeds and leaves,[973] but also in the juices of roots and tubers,[797] in various fruits, as bananas,[79] in the wood and bark of trees,[227] in germinating pollen grains and in the secretions of *Ficus elastica*, *Carica papaya* and *Papaver somniferum*. The leguminosae, particularly *Phaseolus* and *Pisum*, are especially rich in diastase.[1147] It has been suggested that there are at least two diastases in plants, distinguished as *trans-*

location diastase, or amylase which is present in the leaves and reproductive organs, and *secretion diastase*, or amylopectinase, which is formed during seed germination at the expense of the protoplasm and cell nuclei.[200, 495] The first enzyme acts more readily on soluble starch and hydrolyzes the freshly formed starch during its process of assimilation in the chloroplasts; this enzyme is present in all parts of the plant, especially in seedlings and leaves. The second enzyme acts more readily on starch paste, and occurs in the seed during the process of germination. The failure of certain monocotyledons (onion, leek) to form starch in the mature leaf is perhaps due to the lack of one of the enzymes (amylase, dextrinase or maltase) necessary for complete starch formation.[243]

Proteases, chiefly of an ereptic nature, are universally distributed in the plant kingdom; they are very abundant in *Carica papaya* (papaw),[194] *Ananas sativa* (pineapple), and the pitchers of *Nepenthes*. In germinated barley, in *Linum* and in *Cannabis*, there is a proteolytic enzyme which is capable of forming peptone-like substances from coagulated fibrin.[132] This enzyme is absent in ungerminated barley, *Lupinus*, *Pinus* and *Amygdalus*. The cotyledons of *Lupinus* as well as *Ricinus* seedlings, when extracted with glycerin, contain an enzyme which hydrolyzes fibrin to amino acids, including leucine and tyrosine.[494] The reaction is at its optimum at 40°C. in a weakly alkaline medium. The phenol-gelatin method is often utilized to demonstrate a proteolytic enzyme in germinated seeds,[435] such as is produced by the seedlings of *Lupinus*, *Faba* and *Ricinus*.[224] This plant enzyme can be purified by precipitating a glycerin extract with alcohol; its tryptic nature is attested by the formation of amino acids. The optimal reaction is weakly alkaline; 0.2 per cent hydrochloric acid or 0.1 per cent sodium carbonate inhibits plant protease.

Oil bearing seeds, such as *Cannabis sativa* (hemp), contain a much more active proteolytic enzyme than starch containing seeds;[1227] a sodium chloride extract digests proteins without the formation of amino acids, while a water extract is more peptolytic in character. By extracting the seeds with 10 per cent sodium chloride, filtering, acidifying with acetic acid and filtering off the precipitated protein, the ereptase (in the filtrate) can be separated from the peptase (in the precipitate). The latter can be extracted from the precipitate by washing it with slightly acidulated 10 per cent sodium chloride and then with distilled water. The enzyme in *Phaseolus*,[306] in bananas,[79] and in seeds of *Medicago sativa*[600] is ereptic in nature. Although the pressed juice of ungerminated seeds is usually inactive, the juice of the germinated seeds of *Lupinus*, *Triticum*, *Zea* and *Hordeum* can hydrolyze glycyl-l-glycin, leucyl-glycin and dialanyl-cystin.[6]

A detailed review of the occurrence of proteolytic enzymes in branches of trees can be obtained elsewhere.[288] The formation of proteolytic enzymes by insectivorous plants has been demonstrated by a number of investigators. They are typically peptolytic in character and are similar to pepsin.[312,1266] The secretion of Nepenthes is capable of hydrolyzing coagulated fibrin but not glycyl-l-tyrosine.[3] However, the ability of *Utricularia vulgaris* and other plants to digest the bodies of insects, birds and small mammals may depend upon the action of bacteria and not on an enzyme secreted by the plant itself.[663] The optimal reaction for the proteolytic enzyme of malt extract is pH 5.8, with a minimal activity at pH 6.8; the enzyme is more peptolytic than tryptic. The proteolytic enzyme of malt extract may really consist of two distinct enzymes, a peptase digesting native proteins and an ereptase acting only upon albumoses, peptoses and peptides.[1227] According to another view, the proteolytic enzyme of malt either stands midway between pepsin and trypsin or is a mixture of two or more enzymes.[288] A weakly alkaline reaction is favorable to papain[1023, 1070] and bromelin.[227] The various other proteolytic enzymes found in different plants have been rather insufficiently studied.

Oxidases may occur in stems, roots, leaves,[780] latex, petals[199, 331] and fruits.[88a] In general, each living and physiologically active cell contains oxidizing enzymes, especially the pigment-forming phenolases. The seat of these enzymes is apparently the cell membrane and the middle lamella.[1294] Specific oxidases are found in some plants, as for example, laccase which acts upon the pressed sap of *Rhus* producing a lacquer;[78] tyrosinase which occurs in *Russula*, tubers of *Dahlia*, beetroot, root tips, etc., and is capable of converting tyrosine into homogentisic acid; and the olease of olives, which oxidizes fats into simple fatty acids. The oxidase action of the individual organs of various plants may be different.[219] Oxidase and peroxidase are present in potatoes, carrots, beets, turnips and cabbages.[422] Oxidase also occurs in apples, pears, quinces and other fruits, in germinating seeds, in tea[322] and especially in the leguminosae and various mushrooms. The oxidase content of potatoes increases during the process of ripening.[37] The oxidases in old seeds remain active even after the seeds have lost the capacity of germination.[600]

The rôle of enzymes in plant respiration is discussed in detail elsewhere (p. 231).

Peroxidase is present in various plants[944,1227] especially in potato tubers and horse radish. The peroxidase of horse radish is apparently a nitrogenous glucoside which contains over 30 per cent of pentoses, an equimolecular proportion of hexose, 5.5 per cent ash and 0.46 per cent iron.[1275] The freezing of plants results in an increase in the peroxidase content.[530]

Catalase is so widely distributed in the plant and animal kingdoms[121] that the earlier investigators assumed that it was an essential property of the living cell. Various leaves, such as those of clover and *Picea*, certain seeds, such as apple and peach, and potatoes[37] are especially rich in catalase. The catalytic power of plant tissues with reference to H_2O_2 tends to increase under conditions which favor vegetative growth.[546] The catalase content of healthy plants is more or less constant but may be reduced in those which are diseased.[531] In the case of fruit tree tissues, a relatively low catalase activity seems to accompany a nutritive condition in which the ratio of nitrogen to carbohydrates is very low: the higher the nitrogen content or the higher the proportion of nitrogen to carbohydrate, the greater is the catalase activity. The physiological response of plant tissues to various conditions of culture and treatment can be conveniently determined by the catalytic action of the tissues.[546]

Pectase is widely distributed in the plant kingdom, occurring in potatoes, clover, alfalfa, beets, tomatoes,[128] kale, turnip, and malt.[628] *Invertase* is present in various fruits;[1228] the invertase content of banana peel increases 40 to 100 per cent on standing;[798] this augmentation is later followed by a decrease. *Rennet* or *lab* is produced by a number of plants in which proteolytic enzymes are formed as, for example, in the juice of the pineapple, of *Carica papaya*, and especially of the fig, *Ficus carica*,[248] in the secretion of *Drosera*, *Pinguicula* and *Galcium verum*, and in the seeds of *Datura*, *Pisum*, *Ricinus* and *Lupinus hirsutus;* also in *Medicago*, *Plantago* and *Ranunculus*.

Lipase can be demonstrated in the germinating seeds of *Ricinus communis* and other Euphorbiaceae and in both the dormant and resting seeds of soy beans, *Cucurbita*, *Cocos*,[179,605] flax, hemp, rape, poppy-seed and corn. *Cytase*, which forms mannose, galactose and pentoses from hemicelluloses is found in the seeds of various plants.[889,1022] This enzyme is apparently formed in the aleurone layer and is particularly abundant in grasses, barley, rye, and oats.[200]

Emulsin has been demonstrated in sweet and bitter almonds, in *Prunus laeurocerasus*, in the roots of orchids and in various lichens. *Myrosinase* occurs in Cruciferae and closely related families. *Phytase* is found in wheat and rice bran.[1179] *Inulase* is produced by the tubers of various plants, such as *Leucojum*.

2. Enzymes in Dormant and Germinating Seeds. Most seeds, especially after germination has commenced, contain a variety of enzymes; for example, amylase, a peptolytic enzyme, lipase, urease, emulsin and peroxidase are present in soy beans, but invertase and protease are absent.[1176] The important enzymes required by the germinating embryo are present in small quantities in resting seed, in a latent condition. They are usually insoluble in water and become activated during the process of germination. By extracting malt with water and precipitating with alcohol, Payen and Persoz,[956] in 1833, were the first to demonstrate that a "*diastase*" was concerned in the saccharification of starch in germinated barley. A similar agency was recognized somewhat later in germinating potatoes, *Ailanthus* branches and other plants. Diastase is also present in small amounts in starch-bearing dormant seeds and to a lesser extent in those which are oil-bearing; these include the seeds of *Phaseolus*, *Pisum*, *Lens*, *Hordeum*, *Secale*, *Triticum*, *Avena*, *Zea*, *Linum*, *Cucurbita* and *Ricinus*.[1306] The seeds of *Medicago sativa* contain diastase and emulsin, but no invertase.[600] During the process of seed germination, before the roots and leaves are formed, the young embryos depend largely for their nutrition upon the substances stored in the seed. These substances are usually in the form of starch. Since this must be transformed into lower carbohydrates, before it can be utilized, diastase plays an important part in seed

TABLE XII

Decrease in starch content of Phaseolus multiflorus during growth[35]

PERIOD OF GROWTH	DRY WEIGHT OF 100 SEEDS OR PLANTS	STARCH CONTENT
days	*grams*	*grams*
0	116.95	62.07
7	98.50	53.84
9	99.71	52.40
12	84.34	34.49
15	77.89	20.18
19	105.66	16.40
23	133.55	14.61

germination. The changes which occur in the starch content of *Phaseolus multiflorus* during its process of germination in the soil are depicted in Table XII. This decrease in starch content with the advance in the growth of the plants is due to the activity of the developing diastatic enzyme. During the process of germination, the enzyme content of the seedling greatly increases, reaching a maximum in six to eight days in the case of wheat; catalase may reach its maximum in three to four days.[71] Oxygen plays an important rôle in the formation of enzymes in germinating seed.[919] The distribution of diastase in the various parts of barley grains and plants is illustrated in Table XIII. The diastase content commences to increase as soon as the seeds begin to swell. The diastase content of the young plant may be nearly one thousand times greater than that of the original seed.[1147] The maximum is attained at the time of the formation of the first leaves. In the case of barley, the maximal diastase production is reached after eleven to twelve days of growth;[361] the diastase content then begins to diminish. The course of the diastase formation by *Phaseolus vulgaris* is expressed in Table

TABLE XIII

The distribution of diastase in barley[200]

MATERIAL FROM 50 BARLEY GRAINS AND PLANTS	DIASTASE CONTENT EXPRESSED IN GRAMS OF CuO FORMED
Endosperm layer, containing embryo	9.80
Endosperm layer, not containing embryo	3.53
Rootlets	0.07
Plumules	0.05
Scutella	0.55
Complete plants	13.99
Ungerminated seed	2.49

TABLE XIV

Diastase formation by Phaseolus vulgaris grown at room temperature[1147]

DAYS AFTER PLANTING	UNITS OF ENZYME			
	In bean, then in plant	Hypocotyl	First leaf	Second leaf
0	0.0027			
11	1.27	0.770	2.72	
19	0.738	0.308	1.70	
27		0.575	0.620	0.601

XIV and Figure 1, one enzymatic unit being the number of grams of starch which one gram of leaf can transform in twenty-four hours. During the development of diastase in germinating cereal grains the starch-liquefying and sugar-producing powers do not always proceed parallelly;[352,361] the latter may quickly reach a maximum and then begin to fall, while the former may develop slowly and reach a maximum later. In most instances, however, there is a constant ratio between the two enzymatic phenomena.[1147]

The cytolytic enzymes (*cytases*) should be distinguished from diastase.[200] The former dissolve the cell wall of the endosperm during the process of germination; this process of dissolution can be studied microscopically.[502] Some seeds, such as lupine,[889] contain very much more cytase than diastase. Cytase is obtained by the same methods as diastase and so far the two enzymes have not been separated; the former is destroyed by heating at 60°C. for thirty minutes. The reserve celluloses of different plants are digested in different degrees by the same cytase as, for instance, barley cytase.

The formation of diastatic enzymes in the barley grain has been ascribed to a secretion, (1) of the endosperm itself, (2) of the aleurone layer and (3) of the scutellum. According to one view, cytase, diastase and probably also protease are secreted by the epithelial layer of the scutellum and they first affect the adjacent cells.[779a] The breaking down of the cell walls by cytase always precedes the action of diastase. Further information on the secretion and localization of diastase and cytase in barley and other grains may be obtained elsewhere.[288,779a] The quantity of diastase in the plant is dependent to a large extent upon the size, vigor, and condition of the epithelial layer. The secreting area in any seed is greatest at the time the scutellum extends well over the edges of the adjacent endosperm. The most abundant secretion is in well-matured, well-cured grain. Small barley grains produce the greatest amount of diastase while the large grains give the highest yield of malt extract. Barleys rich in nitrogen are usually also rich in diastase. As pointed out above, two diastases are probably present in the barley grain: one of trans-location, which is rather weak; and the other of digestion, which is capable of great activity. The endosperm is incapable of self-digestion except for the slight action of the diastase of translocation. In addition to diastase and cytase, barley and malt contain also various other enzymes, especially emulsin, cellase and trehalase.[570]

"Malting" consists first in the breaking down of the cell walls of the endosperm of the barley grain by the cytase, thus leaving the starch grains exposed to enzymatic action, and, second, in the abundant formation of diastatic and proteolytic enzymes. The object of malting is to subject all parts of the endosperm to the initial effects of the enzymes with as little loss as possible.

Lipase is found in various seeds, particularly in oil-bearing seeds, such as castor oil bean and soy bean. This enzyme plays an important rôle in the germination of oil-bearing seeds; it is not present in resting castor oil seeds, but develops if the seeds are ground and kept in acetic acid at 35°C.[494] The enzyme is probably present in the seed in the form of a zymogen, which is activated by the acid. Lipase is probably produced by the endosperm and not by the embryo; its amount increases during the process of seed germination.[308] Lipase has been demonstrated in linseed, rape seed, flax, hemp, corn and the opium poppy.

The *catalase* and *oxidase* content of seeds depends upon the dormancy, age, vitality and respiration of the seeds.[38] The catalase content of grain increases with its capacity of germination;[283,877,1007] the addition of calcium carbonate to the soil increases the catalytic power of germinating seed.[71] The enzyme often remain active after the seed is dead.[1265] In dead seeds the catalase activity decreases if the seeds are kept in water at 32°C. for twelve hours or at 52°C. for two hours, while in seeds which are alive no such diminution in catalase activity occurs.[301a] This is not true, however, of trees. Catalase is not merely a by-product of plant metabolism, but is formed as a result of the stimulus of the substance which it attacks; its amount cannot always be used as an index of metabolism.[1015]

Seminase, or the enzyme acting upon mannans, is formed abundantly by various seeds;[175–178] the seminases of different seeds are not identical. A milk coagulating enzyme has been found in the berry of the horse nettle.[153] The enzymes of dry seeds may retain their power for a considerable length of time. It has been shown, for example, that diastase, protease and ereptase of wheat and barley seeds retain their activity for twenty years.[1265] Urease preparations made from soy beans eighteen years old have been reported to be almost twice as powerful as those from beans only one year old.[1234] The addition of amylase to old tomato seeds greatly increases their germinating power.[61] The heating of dry grain to

100°C. has no effect upon the enzymes, but a temperature of 130°C. for one hour destroys the enzymes of dry oats.

3. Enzymes in Leaves. The presence of diastatic enzymes and emulsin in the leaves of plants has long been known;[183] they possess much more diastase than is required to transform all the starch that the leaves could ever contain. At first the amount of diastase was thought to be increased at night and diminished during the day. The speed of the transformation of starch in the leaves was regarded as an index of the amount of enzyme present; the greater the hydrolysis of starch, the larger the amount of enzyme. However, there are no consistent differences in the diastatic action of leaves at night and during the day and no apparent correlation between the diastatic activity and the amounts of starch and sugar present in the plant.[1147] The opening and closing of the stomata of the leaves is controlled by the diastatic enzymes.[1255] The latter transform the osmotically inactive starch into osmotically active substances, chiefly sugar, and as a result there is a change in the osmotic properties of the cell sap and in the turgor of the plant, which influences the opening and closing of the stomata. Leaf diastase consists of two components, an inactive organic "pro-enzyme" and an activating "co-enzyme." The former is influenced by light and the carbon dioxide concentration of the sap and the latter by the ions of various salts. The reaction optima, maxima and minima of plant enzymes are not as definite as those of animal enzymes.[1147] Active diastase is present in the protoplasts of the leaves of *Robinia*, *Platanus*, and *Ricinus*, even after they have been subjected to autolysis at high summer temperatures for one to twenty-three days.[943] More enzyme is present in young than in old leaves and none in fallen, dead leaves. The diastase in the leaf may be almost wholly in combination with the protoplast.

In addition to diastase, the leaves of plants often contain several other enzymes.[289] For example, alfalfa contains diastase, emulsin, rennet, a peptolytic enzyme and peroxidase; an insoluble lipase may also be present. Invertase and true protease are lacking.[600] The absence of invertase in the leaves of marigolds may possibly explain the failure of its roots to enlarge; for in sugar beets, the cane sugar formed in the leaves migrates to the roots as invert sugar and is there transformed again into cane sugar and stored.[1202] The cotyledons of *Lupinus albus* seedlings and the cotyledons and endosperm of *Phoenix dactylifera* contain a strong cytolytic and a feeble amylolytic enzyme.[889] The proteolytic enzyme in the leaves of *Spinacia, Brassica, Castanea,* blossoming heads of *Daucus carota,* etiolated seedlings of *Phaseolus mungo,* seeds and seedlings of *Cucurbita macima* and seeds of *Cucurbita pepo* and *Phaseolus vulgaris* is ereptic in character.[306] However, the leaves of *Drosera rotundifolia* contain a proteolytic enzyme of the pepsin type but no trypsin or erepsin.[312]

There is no relation between the oxidase activity and the rate of respiration in potato tubers, but a striking correlation exists between respiration and catalase activity.[37] The oxidase activity of the foliage of normally developing potato plants is greatest during the early stages of development; it decreases with the growth of the plants but rises again when plant growth has become practically stationary.[219]

4. Enzymes in Pollen. Diastase, invertase, catalase, reductase and pectinase have been found in eighteen species of pollen;[952] pepsin, trypsin, erepsin and lipase are only occasionally encountered and the evidence for the presence of cytase is doubtful. Tyrosinase and laccase can not be demonstrated, while zymase has been found only in the pollen of the Siberian crab apple. Rye pollen contains diastase, lipase, protease and catalase.[637]

5. Enzymes in Roots. In addition to the other enzymes of roots which have been previously described, there should be mentioned peroxidase which apparently plays an important rôle in the process of oxidation in roots. It is secreted by the roots into the surrounding soil where it assists in the oxidation of organic matter. Vigorous oxidation can be demonstrated in most productive soils. This peroxidase acts best in neutral or slightly alkaline solutions; the presence of acid and anaerobic conditions is injurious to its activity.[1102]

6. Enzyme Secretion by Diseased Plants. The diastase and oxidase content of diseased potato tubers is greater than that of normal ones.[330,332] The leaves of sugar beets and apple trees which are diseased contain much more oxidase than those which are healthy.[219,1043] Similar observations have been made in curly-dwarf potato plants.[219] An increase in the oxidizing enzymes seems to be more characteristic of the so-called physiological plant diseases, such as the mosaic disease of tobacco, curly top of beets and certain diseases of mulberry trees and spinach, than of those caused by microörganisms.[1009] The plant fungus, *Glomerella rufomaculans*

produces several active enzymes such as diastase, invertase, cytase, inulase, lipase, emulsin, protease, erepsin and amidase. These enzymes are capable of breaking down certain important constituents of the tissues upon which the fungus grows. Tannin is formed as a result of the action of oxidizing enzymes upon certain polyatomic phenols. Injuries produced mechanically or by parasitic fungi may activate these enzymes and cause the production of tannin or tannin-like bodies, which are toxic to the growth of parasitic fungi. The tannin content of fruit varies to a certain extent with the conditions favorable for enzyme action.[276a] The enzymes of the microörganisms which cause plant diseases will be considered elsewhere (see p. 114).

CHAPTER VII

ENZYMES OF MICROÖRGANISMS

Since the report[1224] in 1886 that *B. mesentericus vulgatus* produced cytase, diastase, invertase, protease and rennet, a large number of enzymes have been demonstrated in the culture media in which microörganisms have grown, in the cells themselves and in their spores.[1063]

1. METHODS. The methods for the demonstration of the enzymes of microörganisms must usually be more sensitive than those used in studying the enzymes of higher plants and animals, but the general principles are, of course, the same. If the organisms are grown in liquid media, the enzymes may be separated from the living cells by the filtration of the culture through clay or infusorial earth filters (filtration may remove part of the enzymes) or by killing the organisms either by heating to 55° or 60°C. (in many instances some of the enzymes are destroyed) or by the addition of antiseptics such as phenol, thymol, toluol, chloroform, salicylic acid and sodium fluoride and centrifuging out the dead cells. The enzyme content of the filtrate or supernatant fluid may then be studied. Because of the fact that most bacteria produce only a limited amount of growth products in culture media, it is frequently difficult to demonstrate measurable quantities of enzymes in the media after the bacterial cells have been removed. If the enymes are intracellular they can be extracted from the bacterial cells by some of the methods described for yeast (see pp. 126, 127).

One of the simplest methods for the demonstration of microbial enzymes consists in growing the organisms on agar plates in which various substrates such as blood, casein, fat or starch have been incorporated.[370,371] This plate method has been improved by using a medium containing 0.1 per cent K_2HPO_4, 0.05 per cent $MgSO_4$, 0.05 per cent KCl, 0.001 per cent $FeSO_4$ and 2 per cent agar in distilled water.[281] Carbohydrates, fats or proteins are then added, depending upon the enzyme to be tested. For the study of *diastase*, starch agar is used; halo production indicates diastase formation. This can be confirmed by pouring a solution of iodine in potassium iodid over the plate.[27] *Emulsin* is indicated by the production of a blue color on esculin or by a brown color on arbutin. *Lipase* can be detected by acid production from fat; this is demonstrated by a change in the color of litmus or other indicator added to the agar. For the demonstration of *protease*, the halo production on agar, to which pulverized fibrin has been added, is utilized. For *ereptase*, 1 per cent casein agar is used; for *tryptase*, egg albumen agar; for *amidase*, agar containing 0.5 per cent asparagine and 5 cc. of 2 per cent rosolic acid per liter. The rosolic acid, which is yellow in acid solutions, gives a slight yellow tint to the medium. The liberation of ammonia from the asparagine renders the medium alkaline, and the rosolic acid becomes brilliant red. Diastase formation can also be demonstrated by means of phosphorescent bacteria.[115,116] The latter do not act upon starch, but in a starch medium in the presence of an organism which produces diastase, they become luminous under the influence of the sugar formed. To measure *diastase* formation by certain bacteria, 8 cc. of a four-week old broth culture of the bacteria may be placed in each of two test tubes.[485] One of these is then boiled. Four cubic centimeters of a sterile 0.05 per cent starch solution containing chloroform or toluol are then added to each tube. After twenty-four or more hours' incubation, iodine solution (1 gram iodine, 1 gram KI in 400 cc. water) is added drop by drop to the two tubes and the strength of the blue color in the boiled and unboiled tubes compared.

To demonstrate the formation of proteolytic enzymes by bacteria the following method may be used: 5 to 10 cc. portions of a mixture consisting of 7 per cent gelatin and 1 per cent phenol in water are placed in test tubes and then the liquid to be tested for the presence of proteolytic enzymes (containing 2 per cent phenol) is added.[429–434] Instead of phenol, thymol and other antiseptics may be used. The rapidity of gelatin liquefaction is then recorded. In the case of weak enzymes, days or weeks are required to obtain results. This method has been improved by using water saturated with thymol to which 7.5 per cent gelatin and sufficient cinnabar to color the liquid intensely red have been added.[1101] The mixture is placed in tubes which are then slanted to produce a long surface for the action of the enzyme. The test liquid (with thymol) is then added. To demonstrate the formation of *cellulase* by microörganisms, a medium containing precipitated cellulose has been used.[657] The use of the polariscope has been suggested for the study of peptolytic enzymes.[8,672] Special methods have been devised for the measurement of the other enzymatic activities of bacteria as for instance their proteoclastic action[949] and their ability to ferment lactic acid.[760] A review of the methods for the demonstration of bacterial enzymes has recently been published.[464,1242]

2. ENZYMES OF BACTERIA. *a. General enzymes.* Various alcoholases and acid oxidases, such as lactacidase, are formed by bacteria;[209,211,307,610] the oxidation of higher alcohols by bacteria is probably also a result of enzyme action. *Catalase* is produced abundantly by aerobic bacteria,[230,605,633,853,911,1068,1165] and also by spores.[1063] The time of the maximal production of catalase by bacteria depends upon the type of organism, taking place in twenty-four hours in cultures of *B. prodigiosus* and in twenty-four days in those of *V. cholerae.*[223] The greater the maximal enzyme content of the culture the longer will the enzyme remain active; catalase disappears rapidly from cultures in which the maximal amount is small. Closely related organisms usually possess the same catalytic power. A considerable amount of catalase is formed by *B. proteus* grown on inorganic media containing sodium lactate and one amino acid.[605] Leucine which is essential for the formation of urease can be replaced by other amino acids for the formation of catalase. The addition of toluol to these cultures kills the bacteria, leaving the enzyme unimpaired. $HgCl_2$ acting for a short time will inactivate the enzyme, but KCN will restore its activity. The optimal reaction for bacterial catalase is pH 7.5 to 8.0 and the limiting reactions pH 9.1 and pH 3.1.[1165] The composition of the medium has an influence upon the formation of catalase by bacteria.[1077] The catalytic action of milk is probably accounted for by its bacterial content.[615,680] Catalase formation and bacterial autolysis procede pari passu in a saline suspension of *B. typhosus.*[1226] Pneumococci grown under anaerobic conditions do not produce any peroxide; when brought in contact with air, peroxide is formed, this being a by-product of biological oxidation by means of atmospheric oxygen; the accumulation of the peroxide would become injurious to the organism were it not decomposed by the catalase.[57,59] Anaerobic bacteria produce practically no catalase[667,1068] and possibly this lack of catalase is the reason that oxygen injures anaerobic bacteria.[801] An attempt has been made to classify bacteria according to their catalase production and sensitiveness to H_2O_2.[801]

Cytase, which acts upon hemicelluloses, and *cellulase*, which digests true celluloses, are formed by bacteria;[657,991] the latter is an endoenzyme[991] although there is some evidence that it may also diffuse outside of the cells.[157] Cellulose is digested by several aerobic organisms,[596] by thermophilic bacteria and by an anaerobic bacterium, *B. cellulosae dissolvens,* which occurs among the stool flora.[660] Sulfite lye, a liquid formed during the preparation of cellulose from wood is also fermented by microörganisms.[992] Cellobiose, which is a product of the digestion of cellulose, can be converted to glucose by the action of *cellobiase* produced by bacteria.[443]

Ammonia formation from amino acids and acid amides, due to *desamidase* is a process of common occurrence among bacteria.[123,363,868] Large amounts of *diastase* are produced by *B. anthracis,*[867] *B. subtilis,* [363] *B. megatherium, V. cholerae. M. tetragenes, B. tuberculosis*[434] and pyogenic streptococci[1024] but *B. coli, B. aceticus, B. pneumoniae, B. mallei, Staph. cereus* and *Staph. flavus* form only limited quantities.[429–435] Many bacteria form *emulsin* (β-glucosidase).[431,689,1257] *Hemolysins,* or the enzymes which dissolve red blood corpuscles, are formed by various bacteria, such as *Staphylococci, B. tetani, Streptococci, B. coli, B. pyocyaneus;*[370–371,638,772,803,871,1062,1243] hemolysins were at first considered to be proteolytic enzymes,[1] but later investigations disproved this hypothesis.[802,925]

Some bacteria produce a *gelase* or an enzyme which can hydrolyze agar.[138] Very few bacteria produce *invertase;* among these are: *B. megatherium, B. kiliensis, B. proteus, B. fluorescens liquefaciens,*[432] *B. subtilis, B. mesentericus, B. delbrücki, B. pneumoniae, Streptococcus hemolyticus,*[1167] *B. tuberculosis* and butyric acid bacteria. A higher concentration of endocellular enzymes is produced by pneumococci grown aerobically than by those grown under anaerobic conditions.[870] *Lipase* formation has been demonstrated in cultures of *Staph. aureus, B. pyocyaneus, B. prodigiosus, B. fluorescens liquefaciens* and *non-liquefaciens.*[370,371] Lipase is also produced by *B. cholerae, B. typhosus, B. indicus, B. ruber, Staph. pyogenes albus, Str. hemolyticus,*[1199] *B. lipolyticum, Micr. tetragenes,* bacterial spores[1063] and *B. tuberculosis*[1259] as well as other acid fast bacteria.[658] Sterile filtrates of plain and dextrose broth cultures of *B. typhosus* contain esterase.[658] Further information on the lipolytic enzymes of bacteria may be obtained elsewhere.[836]

Lactase and *maltase* are also formed by various bacteria, the former particularly by the lactic acid group. Bacteria attack the nucleins by means of enzymes (*nucleases*).[1280,976,1069,1085] The formation of *oxidases* by bacteria can be demonstrated by means of agar media.[1105] *B. coli*[1057] and bacterial spores[1063] produce an oxidase, and an aerooxydase has been demonstrated in cultures of *B. tuberculosis.*[106] *Tyrosinase* is formed by bacteria;[476,717] it has been suggested that the action of tyrosinase may be due to a mixture of two enzymes, one produced by an actinomyces and the other by a bacterium, one converting tyrosine into homogentisic acid and the other converting the acid into melanin.[118] *Luciferase* is another example of the oxidases produced by bacteria.[119,475] *B. amyolbacter*, *B. typhosus*, *B. coli*, *B. mesentericus* and other bacteria produce *pectase.*[689,778a]

Proteolytic enzymes are widespread among bacteria as is indicated by the ability of many of them to liquefy gelatin. Attempts have been made to introduce separate names because of the ability of the bacterial protease to attack certain specific substrates, as, for instance, *gelatinase* for the enzyme of gelatin liquefaction and *casease* for the enzyme which liquefies casein, etc., but these are probably not justified. The bacterial proteases usually diffuse into the medium and can be concentrated by filtration or by precipitation with alcohol. Protease is formed by *B. prodigiosus,*[499] *B. anthracis, B. subtilis, B. ramosus, B. tuberculosis,*[320] *B. megatherium,*[434] *Str. hemolyticus,*[1262] *V. cholerae, B. proteus,*[782] *B. pyocyaneus,*[353] *B. diphtheriae*[321] and bacterial spores.[1063] Bacteria form proteolytic enzymes most abundantly when grown in protein media which do not contain sugars. The proteolytic enzymes of the common milk organisms produce four times as much peptone in gelatin as in milk.[949] Very few bacteria produce proteases on protein-free media.[324,433] The enzymes are not preformed in the bacterial cell; their production is dependent on the presence of specific substrates in the medium, for example, *B. cloacae* produces an extra-cellular proteolytic enzyme, the concentration of which depends on the concentration of the medium.[579] The optimal reaction for the protease of *B. pyocyaneus* and *B. prodigiosus* is pH 7.2.[816] The bacterial proteases are classified with trypsin or tryptases because of their sensitiveness to acids, but they are not identical with animal trypsin. The fact that bacterial proteases do not readily digest pure proteins suggests their ereptic nature.[281,1162a] However, the proteases of different bacteria vary and *B. tuberculosis* produces both tryptic and ereptic enzymes.[280] The proteolytic enzymes of diphtheria bacteria are probably of importance in the growth and toxin formation of these organisms.[321] An extract has been obtained from the bodies of tubercle bacilli which inhibits the action of trypsin and leucoprotease.[617]

Reductases. The ability of milk to reduce methylene blue[871] is generally conceded to be due to the bacteria and leucocytes found in milk. The reduction of dyes by bacteria is a function of the bacterial plasma which does not diffuse into the liquid and appears to be a property of all bacteria;[147,488] the rapidity of reduction depends on the number of bacteria and temperature.[1151] The connection between this phenomenon and oxidation processes is discussed elsewhere (p. 243).

The reductase test has been utilized for estimating the approximate number of bacteria per cubic centimeter of milk;[468,615] 1 cc. of a standard solution of methylene blue is added to test tubes, 2 by 20 cm., containing 40 cc. of well shaken milk. These tubes are then corked, shaken thoroughly and placed in a water bath at 38°C. If the methylene blue is not discolored in five and one-half hours, less than 500,000 bacteria per cubic centimeter are assumed to be present. If the dye is decolorized in less than five and one-half hours but not in less than two hours, it usually contains $\frac{1}{2}$ to 4 million organisms per cubic centimeter. If the dye is discolored in less than 2 hours but not in less than twenty minutes, it contains 4 to 20 millions. If the dye is decolorized in less than twenty minutes, there are more than twenty million organisms per cubic centimeter.

Fresh milk to which formaldehyde has been added also reduces methylene blue.[1081] This reduction, or the Schardinger indirect reduction test, is believed to be produced by an aldehyde reductase which is associated with the fat globules[1150] and probably consists in the hydrolysis of water by an oxidizable substance which, with the interaction of a catalyzer, forms a labile, strongly reducing compound with the hydrogen of the water.[64] Sterile broth extracts of pneumococci decolorize methylene blue, but sterile extracts prepared by washing the pneumococci do not. The ability of the sterile broth extracts of pneumococci to reduce methylene blue is destroyed by exposure to the same temperature as that which has been found to destroy the peroxide-forming activity of these extracts. It has been suggested that these two properties are similar in origin.[57–59] Sterile filtrates of aerobic cultures of the pneumococus which contain H_2O_2 can convert catalase-free solutions of hemoglobin into methemoglobin.[894] A new method has recently been described for the study of the oxidase production by pneumococci and streptococci, which is of practical value in their isolation in pure culture.[428]

Rennet is formed in large amounts by many bacteria, including *B. proteus*,[738] *B. prodigiosus*, *B. fluorescens liquefaciens*, *B. pyocyaneus*, *B. amylobacter*, *V. cholerae*.[482,533] One rennet (lab) unit has been defined as that amount of enzyme which completely coagulates 2 cc. of milk containing 1 per cent phenol, at 35°C. in twenty-four hours;[363] purified preparations of bacterial enzymes (cultures grown in milk) contain from 150,000 rennet (lab) units for *B. prodigiosus* to 380,000 for *B. fluorescens liquefaciens*.[534] Rennet is also formed by bacteria in casein-free media, indicating that the formation of this enzyme is a normal function of the cell.[238] It has been demonstrated in a number of bacterial cultures which had been filtered through porcelain.[274] By the use of antisera, bacterial rennet has been found to differ in different species of bacteria and also to differ from animal rennet.

Urease is produced in comparatively large amounts by urea bacteria, as well as by other organisms which are capable of decomposing urea.[842] The enzyme content of a culture depends on the age of the culture and the conditions of growth; the presence of leucine and one other amino acid is required for the formation of urease both by urea bacteria and by *B. proteus*, while no enzyme may be formed on certain urea media on which the organisms thrive well.[605,606] According to some reports urease is a soluble enzyme[842] while to others it is an endoenzyme.[117] It is readily precipitated by alcohol, the precipitate remaining active for several years. A detailed review of the formation of urease by microörganisms may be obtained elsewhere.[754] Bacteria can also produce a *zymase* (lactolase) which converts hexose into lactic acid.[211,559]

It is probable that the biological fermentation tests by which various organisms are usually differentiated from one another depend upon bacterial enzyme secretion; the reason that *B. coli* produces acid and gas when grown in a lactose medium and that *B. typhosus* does not, is probably due to the secretion of lactase by *B. coli* and its absence in *B. typhosus*. In many instances, the repeated cultivation of a bacterial strain in a medium containing a carbohydrate, which that culture does not usually ferment, may cause the culture to acquire the ability to ferment it. For instance, the cultivation of so-called non-xylose fermenting typhoid bacilli in a xylose medium produces a rapidly fermenting strain.[1193] However, this phenomenon is more probably due to the increased multiplication under favorable conditions of a few organisms which can ferment xylose slowly, rather than to the spontaneous generation of an ability to secrete xylase. A review of enzyme production by microörganisms has recently been published.[1242]

b. Bacteriolytic enzymes of bacteria. Some bacteria produce enzymes which possess bacteriolytic properties as, for example, *B. pyocyaneus* which forms *pyocyanase.*[189,216,353,378,379] This enzyme has been classified with the nucleases; it is heat resistant and is destroyed only after thirty minutes at 120°C. Filtrates of cultures of *B. subtilis* can dissolve pneumococci, typhoid and colon bacilli and cholera vibrios and if cultures of these organisms are inoculated with *B. subtilis*, a clearing of the medium results.[890] After a culture of *B. anthracis* has autolyzed, the autolysate can dissolve a fresh culture of anthrax bacilli.[779] Bacteria of the tyrothrix group dissolve living as well as heat killed cultures of *B. typhosus*, *B. coli*, *B. proteus* and certain cocci;[1051] several strains of *B. mycoides* can dissolve other bacteria and cells.[857] There is no evidence of proteolysis when immune serum and complement cause bacteriolysis of typhoid and colon bacilli, although proteolysis is demonstrable when trypsin acts upon the same organisms.[617]

c. Bacteriophage, bacteriolysant, Twort-d'Herelle phenomenon. Although it has not been definitely proven, it is probable that the bacteriophage or bacteriolysant or the Twort-d'Herelle phenomenon is an example of the action of the bacteriolytic or autolytic enzymes of bacteria.[76,302,699,767] Twort[1208] found that the filtrate of a staphylococcus culture even when diluted one to one million would dissolve and kill most of the organisms in fresh staphylococcus cultures (on agar). The remaining cocci could afterwards overgrow these areas of degeneration. This filtrate could be passed on to numerous generations by transferring it to successive staphylococcus cultures, incubating and then refiltering. d'Herelle[323] inoculated 2 to 3 drops of a stool from a dysentery patient in broth and filtered this culture through a Chamberland filter. A trace of this filtrate would dissolve and kill broth or agar cultures of *B. shigae*. A loopful of the filtrate obtained by filtering this lysed culture through a Chamberland filter would again dissolve a fresh culture of *B. shigae*. This bacteriolytic substance has later been produced by several different methods: The injection of a culture of *B. coli* intraperitoneally into a guinea pig produces a peritoneal exudate which can dissolve colon bacilli.[164] This lysis is not complete and a few colonies survive. If these surviving organisms are inoculated into another colon culture, lysis again results. The bacteriolytic action can in this way be repeated indefinitely. Extracts of the small intestine and liver of a guinea pig are lytic for typhoid bacilli.[699] This lytic principle can be transmitted by passage through successive typhoid cultures. Filtrates of broth cultures of recently isolated or old laboratory strains of *B. dysenteriae* (*Shiga or Flexner*) are in many instances bacteriolytic for Shiga and Flexner bacilli.[76,302] Broth cultures of staphylococci and other organisms undergo almost but not complete autolysis within a few days if kept at 37°C. in sealed tubes.[612] This autolysis is probably the same as that involved in the Twort-d'Herelle phenomenon. The substance responsible for the bacteriolytic action of tears and other body fluids, to which the name of lysozyme has been given,[446] is probably another example of a bacteriophage. The lysozyme titer of the blood and tears of rabbits cannot be increased by immunization with *M. lysodeikticus*.[28]

Although d'Herelle's theory[323] that this phenomenon is due to an ultra-microscopic "microbe bacteriophage" cannot at present be disproven, the majority of the observations can best be explained on the assumption that the lytic principle is an enzyme.[302] Temperature, chemicals, the reaction of the media and the concentrations of the lytic agent and substrate (bacteriophage and bacteria) all have an influence on bacteriophagic processes which is similar to that which they have been demonstrated to have on enzymatic phenomena. The optimal reaction for bacteriophagy corresponds very closely to that of trypsin. The fact that cultures of lytic organisms as well as bacteriolytic filtrates do not liquefy gelatin, however, suggests that the bacteriolytic principle is not tryptic in character.[302] The addition of trypsin, or leucoprotease to dysentery cultures, however, will produce bacteriolysis.[74,162,971] The lytic substance responsible for the d'Herelle phenomenon does not resemble malt diastase, *Asp. oryzae* diastase, pepsin, pancreatin or papain,[800] but it has a proteolytic action similar to that of erepsin.[1010]

3. ENZYMES OF MICROÖRGANISMS CAUSING PLANT DISEASES. *Rhizopus* cannot infect sweet potatoes if the skin is unbroken;[527,528] infection is possible only after the middle lamella has been dissolved by means of a pectinase secreted by the growing hyphae. All infections occur in wounds in which there is some tissue upon which the fungus can obtain a saprophytic start. A pectinase is formed

by the fungus, during the growth of the mycelium upon the dead cells. This enzyme, on coming in contact with the living cells of the host, dissolves the middle lamella. The cells then die and thus provide a suitable substrate for the further development of the fungus. Infections caused by *Botrytis cinerea*[201] and *B. carotovorus*[628] behave similarly. *B. carotovorus* which causes the soft-rot of carrots, celery and other vegetables, produces a *pectinase*, that dissolves the middle lamella of the parenchymatous cells of the plants which it attacks.[628] The enzyme secreted by the bacteria penetrates into the tissues and completes the action considerably in advance of the invasion of the organism. The cell wall, however, remains unchanged unless it is dissolved by cytases which are produced by other bacteria.[982] The elaboration of cytase occurs during the protoplasmic activity which is associated with the growth of the hyphal tip of the fungus. In addition to pectinase and cytase, various parasitic fungi, such as *Botrytis* and *Glomerella rufomaculans*, produce amidase, diastase, emulsin, ereptase, inulase, invertase, lipase, protease and oxidase, both in liquid media and in the infected fruit.[1009] Further information on the enzymes formed by fungi which are pathogenic for plants may be obtained elsewhere.[144,204,1209]

4. Enzymes of Molds and Other Fungi. The study of enzyme formation by fungi is less difficult than that by bacteria because of (1) the abundant growth of mycelium formed on suitable media by the great majority of fungi and (2) the ease of separating the mycelium from liquid cultures by mere filtration through paper. The determination of the influence of the composition of the medium upon enzyme formation as well as the qualitative and quantitative separation of the enzymes secreted in the medium from those retained in the mycelium are relatively simple. The acetone-ether method is very convenient for the demonstration of the enzymes of fungus mycelium. By the use of this method it has been found that P. *camemberti* produces amidase, diastase, emulsin, erepsin, inulase, lactase, lipase, maltase, nuclease, protease and raffinase.[339] *Asp. oryzae* forms amidase, diastase, catalase, inulase, invertase, lactase, lipase, maltase, protease, rennet and sulfatase.[892] A variety of enzymes are produced by *Asp. niger*,[463,1255a] *Myxomycetes*,[1103] the wood destroying fungi[1096] and the higher fungi.[1320] The *Saprolegniales* form many enzymes, including amylase, cellulase, inulase and pectinase but not catalase, lipase, oxidase, peroxidase and urease.[380a] The *Hymenomycetes*, which cause mycorrhiza in various

trees, produce active proteolytic enzymes; the amount of the different enzymes varies with the organism and depends on their specific adaptation to organic nitrogen compounds; this enables the trees to utilize the nitrogen in complex organic forms.[804] A detailed review of the enzymes formed by fungi has recently been published.[1242]

Catalase is formed by a number of fungi.[340,869,990] *Cellobiase* is produced by *Asp. niger*, *Asp. wentii*, *P. purpurogenum*, *Mucor mucedo* and other fungi.[129a,989] *Cellulase* formation by soil organisms has been demonstrated.[561] *Cytase* is produced by a number of fungi, including *Asp. oryzae*, *Asp. wentii*, *P. pinophilum*, green *Penicillia*, *Rh. nigricans*, various *Mucorales*, certain pathogenic fungi and several *Actinomycetes*. *Desamidase* and *desaminase* formation by fungi has been reported.[988,1136] The formation of *diastase* by the *Asp. oryzae* group of fungi is of great economic importance; the enzyme is obtained by growing *Asp. oryzae* on rice or wheat bran for 36 to 48 hours, then extracting the enzyme by percolating the mass of growth with water. The abundant diastase formation is characteristic not only of *Asp. oryzae* but of a large number of *Aspergilli*, *Penicillia* and *Mucorales*. The longer the conidiophores of the *Asp. oryzae-flavus* group of Aspergilli the greater is the diastatic power of the organism; the shorter the conidiophores the greater is their proteolytic power.[930] Among the Mucorales, *M. rouxii*, *M. boidin* and allied species possess greater diastatic power.

Emulsin is widely distributed among the fungi, including Aspergilli (*Asp. niger*, *Asp. oryzae*, *Asp. wentii*, *Asp. terricola*), and Penicillia (*P. italicum*, *P. luteum*, *P. camemberti*).[997] This enzyme may be obtained by growing *Asp. niger* in Raulin's solution until the time of spore formation, then replacing the liquid part of the culture with distilled water.[177] The emulsin will gradually diffuse into the water. The presence of ammonium nitrate in the medium completely represses the formation of emulsin by *Asp. niger*. The enzyme is abundantly formed, however, if the medium containing NH_4NO_3 is replaced by distilled water. *Gentianase* and *Gentiobiase* are also produced by *Asp. niger*.[169–176] *Inulase* is formed by *Asp. niger*, *P. camemberti*, various green *Penicillia*, certain species of *Rhizopus*, *Glomerella rufomaculans* and other fungi. The production of inulase by *Asp. niger* reaches a maximum at the period of sporulation of the fungus and then begins to diminish rapidly.[1313] *Invertase* can be obtained from a large number of fungi, including various Aspergilli (*Asp. niger*, *Asp. oryzae*), Penicillia (*P. camemberti*, *P. glaucum*), Fusaria and Monilia. Several members of the Mucorales group and also certain actinomycetes are incapable of producing invertase. The −strains of *Mucor racemosus* form invertase while the + strains do not.[686] The formation of this enzyme by fungi is an important factor in the spoilage of sugar.[683] The inversion capacity or invertase content of a green *Penicillium* is greatest on the fourth day of growth, at a temperature of 16°C.[405] The conidia of the fungus have only one third of the inversion capacity of the mycelium. The highest inversion capacity is exhibited by cultures grown in solutions of cane sugar and galactose. The *Penicillium* produces only one-tenth as much invertase as bottom yeast and one-sixth as much as top yeast, as is illustrated in Table XV. The optimal reaction for *Penicillium* invertase is pH 4.0 to pH 6.0, i.e., between that of yeast and intestinal invertase,[406] as shown in Figure 5.

TABLE XV

Comparison of invertase production by various fungi

	INVERSION CAPACITY	RELATIVE INVERSION CAPACITY
Bottom yeast	0.15	100
Top yeast	0.09	60
Pen. glaucum	0.015	10
Asp. niger	0.0032	2
Asp. flavus	0.002	1.4

Lactase is formed by several species of *Rhizopus*, *M. rouxii*, various *Penicillia* and *Aspergilli*.[339,989] *Lipase* is produced by a large number of fungi, including *Asp. niger*,[231,1083] *Asp. terricola*, *P. roqueforti*, *B. camemberti*, green Penicillia, *Monilia sitophila*, certain species of *Rhizopus* and other Mucorales. The formation of lipase by fungi is influenced quantitatively by the source of the carbon in the medium, triolein giving rise to the greatest production.[1083] Lipase formation reaches a maximum in two days of growth. *Maltase* is usually an endoenzyme and is formed by a number of fungi, including the group of *Asp. niger* and *Asp. oryzae*, various *Mucors*,[989] *Penicillia* (*P. camemberti*), *Monilia sitophila*, *Allesheria gayoni* and others. The enzyme is formed even when the fungus is grown on synthetic media containing various carbohydrates.[339] *Melezitase* is also formed by *Asp. niger*.[169–176] *Nuclease* is produced by *Asp. niger* and *P. glaucum* in media containing nucleic acid as the source of nitrogen and phosphorus;[598] however, *P. camemberti* makes an endocellular nuclease even when grown in a nucleic acid free medium.[339] *Oxidase* formation by fungi has been studied chiefly in connection with certain pathogenic organisms, such as *Botrytis cinerea.*[1305] *Pectinase* is formed by certain species of *Rhizopus, M. hiemalis, Botrytis cinera, Cladosporium herbarum* and others. The formation of *phytase* by fungi has also been demonstrated.[342]

Protease. *P. glaucum* grown in a 6 per cent gelatin solution rapidly liquefies the gelatin;[517] a glycerin extract of the fungus, prepared soon after liquefaction of the gelatin has begun when only a few spores have been formed, contains a proteolytic enzyme which liquefies neutral solutions of gelatin. An extract of *Asp. niger* grown in Raulin's medium, prepared by triturating the mycelium with sand and chloroform water, converts fibrin and coagulated egg white at 40°C. in a slightly alkaline medium to peptones.[168,457] The proteolytic enzymes of *Actimomycetes* are also somewhat tryptic in character;[866] however, the proteolytic enzymes of fungi are more closely allied to erepsin than to trypsin, since their action upon native proteins is very slight, while peptones are readily digested.[339,1297] It is, of course, possible that different fungi contain different proteolytic enzymes.[7] Various fungi also form *peptolytic* enzymes capable of hydrolyzing artificial peptides.

Raffinase is produced by *Asp. niger* and *P. camemberti* especially if lactose is the source of carbon.[339] *Rennet:* Various fungi, including *Aspergilii, Penicillia* and some species of *Rhizopus* produce a milk coagulating enzyme, which resembles calf rennet although it is not influenced by calcium chloride. *Sulfatase* is formed by *Asp. orzyae.*[887] *Tannase* is produced by *Asp. niger* and green *Penicillia* in synthetic solutions (Czapek's) containing tannic or gallic acid.[669] Although the intensity of tannase formation by *Asp. niger* apparently depends upon the tannin content of the substrate, this organism can secrete tannase even when cultivated on a substrate which does not contain tannin.[666] *Trehalase* is produced by various Aspergilli (*Asp. niger*), *Penicillia, Monilia sitophila, Allescheria gayoni* and other fungi. The formation by fungi of enzymes which digest uric and hippuric acids has also been demonstrated.[343,346] *Zymase* is also formed by several fungi, e.g., *Monilia candida* and various *Mucorales* when grown under anaerobic conditions. If, after three days' growth in a modified Raulin's medium, the mycelium of *Asp. niger* is submerged and anaerobic conditions produced, alcohol and CO_2 are formed from the dextrose.[635,686]

5. Enzymes of Yeasts. Yeasts form various enzymes; some of these, such as invertase and zymase, have been studied very extensively because of their wide practical application and of their great theoretical importance in connection with the chemistry of enzymatic phenomena. Comparatively large quantities of carboxylase, catalase, invertase, maltase, melibiase and zymase are present in fresh yeast; endotryptase, oxidase and reductase occur in moderate amounts, while amygdalase, emulsin, lipase, rennet and trehalase exist only in small amounts.[100] Yeasts also produce the enzymes arginase,[1137] catalase,[66,397,593–595,728] carboxylase,[520,882–887] desamidase, [362,1111] emulsin,[229,553] gentianase, guanase,[629] lipase,[1030] nuclease,[31] oxidase,[68,522,594] rennet,[716,1002] raffinase and trehalase.[99]

The formation of enzymes which attack polysaccharides is rather limited among yeasts, so that it is doubtful whether many of these organisms can break down cellulose, inulin and pectins; some yeasts form *diastase* and are capable of hydrolyzing starch.[99] Yeasts are very active against the lower carbohydrates. Most of them, with the exception of Torula, *Sacch. apiculatus*, *Sacch. albicans* and wild sugar yeasts, are capable of producing *invertase*, top yeasts being more active in this respect than bottom yeasts. The enzyme is secreted by the organism into the medium. Colloids, as for example gelatin and gum arabic, have an injurious effect upon the secretion of the enzyme.[948] The maximal formation of invertase by yeast occurs in the first 25 hours of growth.[404] As much as 12 per cent of the invertase produced by yeast may be obtained by extraction.[392] The preparation of active invertase from yeast is described elsewhere (see p. 174). *Lactase* is formed by all organisms which are capable of decomposing lactose, as for example, *Sacch. acidi lactici*, *Sacch. kefyr* and various Torulae.[211,1280] Lactase is an endoenzyme. *Maltase* is formed by beer and wine yeasts,[730] *Monilia candida*, *Willia anomalia* and certain *Torulae*;[99] it is usually an endoenzyme. *Zymase:* The discovery of zymase in yeast cells by Buchner[206] was epoch-making. The production of alcohol and CO_2 by yeast cells from dextrose was regarded until that time, as a function of the life activities of the protoplasm of the organism. By grinding up yeast cells with sand, then adding infusorial earth and pressing out the liquid by means of an hydraulic press, Buchner and his associates[208] succeeded in obtaining a cell-free juice which was very active in the production of alcoholic fermentation of fructose, glucose, maltose and sucrose. A detailed study of the nature of this enzyme is described elsewhere (see p. 244).

Protease. The chemical reactions occurring during the autolysis of yeast, which result in the formation of various amino acids, are due to the enzymes formed within the cells.[363,599] These proteolytic enzymes do not diffuse, except in small amounts, outside of living and normal cells. However, if the cells are dead or starved, the enzymes readily pass through the cell wall. Protease can be obtained by the Buchner pressure method; this enzyme has been termed "yeast endotryptase" because of the similarity of its action to that of trypsin.[474] It digests nucleins and various proteins, such as albumin, casein, fibrin, gelatin, and gluten but has less effect upon globulins and serum albumins. Among the products of hydrolysis, there are various amino acids, such as leucine, tyrosine, lysine, aspartic and glutamic acids, but albumoses and peptones have not been demonstrated.[697] The character of these end-products suggests that the enzyme is related to trypsin. The optimal temperature is between 35 and 45°C.; exposure for one hour to 60°C. destroys the enzyme.[966] The optimal reaction is pH 6.0.[314,315] The proteolytic enzyme of yeast consists of more than one enzyme;[1227] there have been differentiated a peptase, whose optimal reaction is pH 4.0–4.5, a tryptase with an optimum at pH 7.0 and an ereptase with an optimum at pH 7.8.[314,315] The maximal action of the mixed enzymes is at pH 6.0. Further information of the peptolytic enzymes of yeast may be obtained elsewhere.[3,450,672]

6. Enzymes of Algae. Various algae, e.g., *Ulothrix cladophora* and *Spirogyra*, produce diastase, invertase and catalase.[1146] The formation of diastase is stimulated by the presence in the culture media of starch, calcium tartrate, and calcium lactate but is decreased by the presence of dextrose, galactose, lactose, maltose and sucrose.[391] Sunlight does not influence the formation of diastase. The optimal reaction is between pH 4.0 and 5.0 in a phosphate mixture. Preliminary treatment of the algae with chloroform or 96 per cent alcohol, for a maximum of three hours, increases the action of diastase. This treatment diminishes the activity of catalase, but drying of the algae stimulates it; the catalase content of *Spirogyra* is also influenced by the temperature of growth.[221] The following enzymes are produced by marine algae: catalase,[514] lipase, nuclease, oxidase, peroxidase and protease; amidase, cellulase, cytase, esterase, invertase,

lactase, and maltase have not been demonstrated;[300] the action of the enzymes that are formed is very weak. *Phormidium laminosum* growing in springs at 74°C. produces lipase and a glycogen-splitting enzyme, but not diastase, invertase and casein-splitting enzymes.[711]

7. Enzymes of Protozoa and Invertebrates. The production of proteolytic and other enzymes by protozoa has been demonstrated.[221,296,855,1220] The catalase content of insects increases with the fertilization of the egg and the development of the organism up to a maximum, then in older insects, decreases.[154,222] Several enzymes are produced by invertebrates, including a pepsin-like and a trypsin-like enzyme, rennet, esterase, maltase, and diastase; inulase, lactase and invertase have not been demonstrated.[152,1024a] A detailed review of the proteolytic, amylolytic, lipolytic and other enzymes of protozoa, arthropods, worms, echinoderms, mollusks, coelenterates and sponges may be obtained elsewhere.[923, 1314]

C. METHODS FOR THE PREPARATION AND STUDY OF ENZYMES

CHAPTER VIII

PREPARATION, PRESERVATION, PURIFICATION AND MEASUREMENT OF ENZYMES

1. PREPARATION OF ENZYMES. In spite of the most painstaking work which has been done up to the present, enzymes have not been prepared as pure chemical substances and even a so-called purified preparation contains a large amount of contaminating material. The classification of enzymes as exo- and endo-enzymes is retained here for convenience, although there is no sharp line of division between them. Some enzymes, such as invertase in yeast cells, are present in the cell in a free condition, soluble in water and not attached to the insoluble constituents of the cell; they can, therefore, be readily isolated by dissolving them out. Other enzymes are present in the cell in an adsorbed condition, as, for example, emulsin in the seed of bitter almonds or peroxidase in grain seed. The adsorption can be overcome by treatment with dilute alkali and other chemical means.

a. Extra-cellular or exo-enzymes. The exo-enzymes of the gastric and pancreatic juices were formerly collected by means of gastric and duodenal fistulae,[110,748,749,953] but at present they are obtained by gastric and duodenal tubes.[148,373,374,696,970] These juices, as well as saliva and various other plant and animal juices and secretions, can be used directly as a source of enzymes or the latter can be precipitated out with alcohol. The extra-cellular enzymes of microörganisms are procured by cultivating bacteria or fungi in liquid media and then removing the cells by centrifugation or filtration. Filtration through paper is sufficient for the fungi and large protozoa, but for bacteria, small flagellates and yeasts, porcelain filters are employed. Several factors, which will be discussed later (see p. 133), affect the passage of enzymes through porcelain filters. The addition of infusorial earth, followed by sedimentation and centrifugation is occasionally used to remove cells from cultures, but some of the enzymes may be adsorbed by this method. In the case of such organisms as *Asp. oryzae*, which grow on cereal products, the enzymes are obtained by percolating the mass of the growth of the organisms with water. The strength of the enzymes can be greatly increased by repeatedly percolating fresh lots of enzyme-containing material with the same enzymatic extract.[1186]

The exo-enzymes can be concentrated by various methods of precipitation and adsorption, as will be shown later. The aqueous extract may, however, be concentrated directly by the removal of the excess of water (or other solvent). For this purpose, the enzyme solution is concentrated at very low pressures and at temperatures of 30°C. or less. This can be accomplished by evaporation in an ordinary vacuum concentrator or by spraying the liquid in a fine stream into an evaporating chamber. A convenient apparatus has recently been devised in which CO_2 is used as the drying gas, in order to avoid the oxidizing influence of air.[1075]

b. Intra-cellular or endo-enzymes. The enzymes of cells, tissues and organs of plants and animals are obtained by pressure, by extraction with various solvents or by the killing of the cell, without destroying the enzyme. All these methods are based upon the destruction of the cell by chemical or mechanical means. The following methods have been devised for the extraction of zymase from yeast:

i. *The Buchner zymase extraction method.*[206–208] Top yeast is thoroughly washed with water and the latter decanted after the yeast has settled. This process is repeated three or four times until the wash water is clear and colorless. The washed yeast is then placed in a bag and pressed in a Buchner press for three minutes at a pressure of 50 atmospheres. The yeast will then be found to contain 50 per cent water. To 1 kilo of this pressed yeast, 1 kilo of sand and 300 grams of diatomaceous earth are added and the mixture thoroughly ground in a porcelain mortar. The ground mass is placed in a strong cloth, previously soaked in cold water, and again placed in a Buchner press. The excess water is pressed out at 50 atmospheres. The pressure is then gradually increased to 300 atmospheres and the pressed out juice allowed to run through a folded filter paper into an ice cold container. Four hundred to 500 cc. of juice are obtained from 1 kilo of yeast. The liquid (juice) obtained by this method is weakly opalescent, low in proteins, and faintly alkaline in reaction. It contains the enzyme (zymase) and can be sterilized by passing through a Chamberland filter. An active liquid can also be obtained by treating the yeast with solid CO_2. The enzyme may be carefully dried, not above 35°C., without decreasing its power, or it may be preserved in glycerin.

ii. *Lebedev's autolysis method.*[714] The yeast cells are thoroughly washed and then dried for two days at 25° to 30°C. The material is mixed with three volumes of water, allowed to autolyze for two hours at 35°C., and filtered through ordinary filter paper. The liquid so obtained is much more active than the Buchner preparation, is glycogen-free, does not become fermented but contains less co-enzyme. A very active invertase can be obtained by a modification[121,266] of this method: 25 grams of yeast are allowed to undergo putrefaction in 150 cc. of water until the reaction becomes acid to litmus, but neutral to methyl orange or *p*-nitrophenol. At this point the invertase activity has reached its maximum. The autolysate is filtered through diatomaceous earth until clear and is then treated with toluol. This preparation will retain its activity for a long period.

iii. *Acetone- and alcohol-ether extraction methods.*[23] Treatment of yeast or other living cells with acetone-ether or alcohol-ether kills the cells but does not injure the enzyme. However, the enzyme thus prepared cannot be kept in solution since it is easily destroyed on moistening. The preparation should be dried over sulfuric acid and then powdered and preserved. It is a more active preparation than that obtained by the pressure method. The zymase of bacteria that produce lactic acid from sugar and the endo-enzymes of molds and bacteria can be obtained in a similar way.[8,209,211,339,1080]

iv. *Heat method.* A commercial yeast product, *zymin,* is prepared by the rapid drying of the cells, followed by heating to 100°C.; this treatment results in the rupture of the cells (in a moist condition, the enzymes are destroyed at 60°C.).

v. *Organ and tissue extracts.* The methods used for the extraction of enzymes from the organs and tissues of animals and higher plants depend upon: (1) direct pressure; (2) extraction by water or other solvents; and (3) freezing and drying in vacuo. Autolysis, at 37°C., should not be used, for if continued too long many of the enzymes are destroyed. An animal organ is first freed from blood, by bleeding the animal from an artery, then washing the system, by introducing saline solution into the jugular vein until the fluid issuing from the artery contains but few red blood cells.[860] The organ (or plant tissue) is then ground up in an ordinary meat grinder or apparatus especially constructed for the purpose. The enzymes may then be obtained by the application of pressure to this macerated mass. The use of diatomaceous earth, which is employed in yeast preparation, is inadvisable organ and tissue extracts for certain enzymes are adsorbed by the earth.

In preparing aqueous extracts of animal organs or plant tissues, distilled water containing an antiseptic, such as alcohol, chloroform, toluol, thymol or glycerol or else dilute solutions of salts, acids or bases, should be used. One per cent chloroform or toluol or 50 per cent glycerol in water are the most suitable solutions. The extracting mixture is allowed to stand at room temperature or in an ice box for a certain length of time, or is shaken in a shaking apparatus for a few hours. Some enzymes, however, are injured by shaking.[1092] The organ may also be frozen completely by means of liquid air, then powdered and dried in vacuo over H_2SO_4.[4684] A grayish white powder is obtained which retains its activity for a long time. This freezing method has also been used to obtain enzymes from bacteria[766] and yeast[327] and is readily applicable to plants. The pulp of ground up animal tissues can also be passed through a sieve, to remove the connective tissues; the mass is then spread out on glass plates in a warm dry atmosphere.[1269] The dried material is next scraped off and ground in a mortar with toluol. This fine suspension is filtered and washed with toluol, in order to free it from the toluol-soluble lipoids and then dried. The powder should be kept in a dry condition. The active enzyme is obtained by extraction of the powder with water, or with weak acid or alkali depending upon the properties and solubility of the enzyme under investigation.

It should always be remembered that enzymes are often bound to the protoplasm, forming part of complex molecules and cannot be extracted completely, so that if the extract is inactive the tissue itself should be tested for the presence of the enzyme.

2. PRESERVATION OF ENZYMES. Enzymes retain their activity for long periods when dry and powdered but are rapidly destroyed when in solution, especially at 50° to 80°C. The reaction of the enzyme solution is an important factor in the duration of its potency. At pH 5.0, invertase remains active for a long time at 15°C., but is rapidly destroyed in alkaline solutions.[406] The various enzymes of organ extracts and pressed juices may destroy one another, as in the action of yeast endotryptase upon zymase. Enzymes are also adsorbed by the gradually precipitating proteins of the solution. Of particular importance is the destructive action of microörganisms

upon enzymes. For this reason, enzyme solutions require the addition of some antiseptic.

Among the chemicals used for the preservation of enzyme solutions are: toluol, chloroform, sodium fluoride, ether, phenol, thymol, oil of mustard and formaldehyde. *Toluol* is the least injurious to enzymes. Since it remains on the surface it prevents invasion of the solution by dust contaminations. When the solution and container are sterile, toluol is quite effective. Sufficient toluol to form a layer over the surface or to produce a one per cent concentration is required. On long exposure toluol destroys pepsin and injures dilute trypsin solutions[363] and also gastric lipase.[1187] *Chloroform* is more readily distributed throughout the liquid, by shaking, than toluol. It evaporates readily and must be frequently renewed. It has also a more injurious effect upon the enzyme than toluol.[554] A mixture of toluol (1 per cent) and chloroform (1 per cent) is often very satisfactory.[12] Lipase is injured by 1.5 per cent chloroform, 6 per cent acetone or iodoform, 0.045 per cent iodine and 0.25 per cent bromine; the last two chemicals practically inhibit lipolytic activity.[947]

Two-tenths to 0.3 per cent sodium fluoride is a valuable preservative; it is soluble in water, does not have a strong precipitating action upon proteins, does not injure most enzymes and, since it does not evaporate, does not have to be renewed. However, it cannot be removed from the enzyme solution as can toluol and chloroform, and, if efforts are made to concentrate the enzyme solution, the sodium fluoride becomes sufficiently concentrated to be injurious to the enzyme. The toxic effect of an enzyme preparation preserved with NaF must also be considered, if it is to be used for food or physiological purposes.

Of the other antiseptics, *thymol*[1091] and *phenol* are very useful in concentrations of 0.1 to 0.2 per cent for the former and 0.5 to 0.6 per cent for the latter. Both, however, and especially phenol, have a destructive action upon some enzymes after a short period of time. They have also the disadvantage of not being removable by evaporation. Gastric and duodenal juices, even though initially contaminated, can be rendered sterile and kept so for several months at 10°C. by the addition of one or two crystals of thymol. Gastric lipase,[1187] rennet and duodenal amylase may be preserved in this way for several months without much loss in activity.[303] Various substrates may also be satisfactorily preserved with thymol. Certain other antiseptics may also be used: 50 cc. of 2 per cent *iodoform* (in acetone) will preserve the enzymes in 2 liters of a culture of cellulose-decomposing bacteria.[991] *Formaldehyde* is occasionally useful;[1130] 1:1000 to 1:2000 solutions have been used for lipase preservation but 1 per cent concentrations are injurious.[947] *Chloral hydrate* is an excellent preservative for gastric lipase.[1187] *Glycerol* has also been recommended.[337a] If too large quantities of the antiseptics are used, the enzyme may be weakened and even destroyed; if insufficient antiseptic or the improper antiseptic is employed, bacterial or yeast growth will take place. The fact that microörganisms do not develop readily in very concentrated solutions is utilized for the preservation of some enzymes. Malt enzymes, as well as those of *Asp. oryzae*, are placed on the market in a highly concentrated liquid form without any disinfectants. The concentration is carried out in vacuo at 35°C. or lower temperatures, so as not to injure the enzyme. In this form, the enzyme can be readily used in bakeries or for other purposes.

Special precautions to eliminate bacterial action should also be taken when titrating the activity of an enzyme. A test should not last longer than two hours, for the multiplication of any bacterial contamination is very rapid after that time; the so-called period of "lag" in bacterial growth occurs during the first two hours.

3. PURIFICATION OF ENZYMES. The enzymes obtained by any of the methods previously described are usually accompanied by foreign substances. These consist chiefly of proteins, carbohydrates and inorganic salts. Various methods have been devised to purify enzymes. One means of purification of diastase is based upon the fact that the enzyme is not a nutrient for yeast. If an enzyme solution is inoculated with yeasts, the proteins and carbohydrates are consumed, while the diastase is left intact.[360,456] This may also be true of enzymes other than diastase. Proteins may also be removed by autolysis or treatment with acid or alkali.[1275] Inorganic salts may be removed by dialysis. However, the enzyme solution may lose its strength as a result of the diffusion of the enzyme or the coenzyme through the membrane. The loss of activity which results from dialysis may in some instances be restored by the addition of such electrolytes as common salt, phosphates or asparagine. These substances do not act as co-enzymes, but they maintain an electro-

lytic medium which is required for enzyme action and also serve as buffering agents.

a. Precipitation. Enzyme solutions may often be purified and concentrated by precipitation. Acetone, alcohol and ether, as well as various salts, are the least injurious precipitants, although for certain enzymes, such as zymase, the alcohol must be removed as soon as possible. An enzyme may be precipitated from an aqueous solution by the addition of an equal or double volume of alcohol or acetone. The resulting precipitate is then washed with 95 per cent alcohol or pure acetone, redissolved in water, filtered and again precipitated. To obtain as pure a product as possible, these processes should be repeated until the addition of alcohol fails to produce any turbidity. The contact with alcohol must be as short as possible and precipitation should not be repeated too often for the prolonged action of alcohol injures the enzyme, making it insoluble and inactive. The final precipitate is washed with alcohol, then with ether and lastly is dried over sulfuric acid. By this method a white powder is obtained which retains its potency for long periods. The liquid enzyme obtained from yeast can be precipitated by adding 400 cc. of absolute alcohol and 200 cc. of ether to 50 cc. of liquid enzyme and then rapidly drying the precipitate.[23] This white powder is quite soluble in glycerin but only partially so in water. The glycerin extract is very active, since the glycerin seems to destroy the injurious endotryptase. The enzyme may be precipitated from yeast juice by the addition of 10 volumes of acetone.[803a] Concentrated *Asp. oryzae* diastase (so-called Taka-diastase) is prepared by precipitating the enzyme from a water extract by adding sufficient alcohol to produce a 70 per cent concentration.[1186] Different concentrations of alcohol are required for the precipitation of various enzymes. All of them are more or less soluble in dilute alcohol. The fibrin ferment is precipitated by 10 to 15 per cent alcohol, while rennet requires 80 to 90 per cent. Lipase and maltase can be precipitated by means of alcohol.[419]

Enzymes may also be obtained in a more or less pure form by precipitation with salts in the process of "salting out." The most important chemicals for this purpose are ammonium sulfate, magnesium sulfate, lead acetate, aluminium hydroxide and uranyl acetate. The salting out of enzymes is usually accomplished by adding a saturated solution of ammonium sulfate to an aqueous solution of the enzyme until 25 to 30 per cent saturation is obtained. (The reaction of the solution must often be adjusted by the addition of a small amount of acid, for such enzymes as the diastase of *Asp. oryzae* which is sensitive to alkalies, or the addition of a few drops of 10 per cent sodium carbonate for other enzymes such as trypsin which is destroyed by acids). The first precipitate which usually consists chiefly of proteins and other impurities is filtered off after twenty-four hours. A sufficient amount of a saturated solution of $(NH_4)_2SO_4$ to make a 60 to 66 per cent solution is then added to the filtrate. The precipitate which is formed contains most of the enzyme and, in addition, some proteins, their degradation products and ammonium sulfate. It is filtered off and suspended in about 5 times its volume of water containing toluol and chloroform, shaken for about thirty minutes and dialyzed in flowing water until the ammonia is removed. Several days are usually required for this process. The enzyme solution is then ready for use or it may be further purified by precipitation with acetone or alcohol[861a,1121] or evaporated in vacuo to dryness.[568a] Further information on fractional precipitation of enzymes is obtainable elsewhere.[213]

Individual enzymes in solutions containing proteins may also be separated from one another by the process of fractional salting out; the diastatic, proteolytic and lipolytic enzymes of the pancreatic juice have been separated by partial fractionization with ammonium sulfate.[749a] Saturation of the enzyme solutions with $MgSO_4$ and NaCl can also be employed; the salt is then removed by dialysis. The reaction of the solution is important for optimal precipitation.[803b] Uranyl acetate (or phosphate) has been employed for the purification of enzyme solutions;[601,602] a saturated solution of this salt is added to a weakly acid or alkaline solution of the enzyme (depending upon the sensitivity of the latter to alkali or acid), until precipitation occurs. The supernatant liquid is siphoned off and the precipitate is filtered. The latter is then suspended in 0.2 per cent sodium carbonate, and allowed to stand a few hours with frequent shaking. Most of the enzyme goes into solution and can be filtered off.

Many of the methods for the isolation of enzymes depend upon the fact that they are carried down in an adsorbed form with amorphous precipitates, especially when the enzyme and the precipitate are electrically charged and of the opposite sign. The acid or alkaline properties of enzymes are useful in their separation, as in the separation of pancreatic lipase, an acid enzyme, from pancreatic trypsin which is alkaline. When sodium phosphate and calcium chloride are added to an enzyme solution, a precipitate of tricalcium phosphate is formed which carries down the enzyme. A diastase preparation which is free from proteins can be obtained by precipitating saliva with calcium phosphate and also a diastase free from trypsin by the similar treatment of pancreatic juice.[263] The addition of a solution of cholesterin in acetone or alcohol produces a similar effect. Adsorbed enzymes are inactive; the precipitant must be separated from the enzyme. In the case of the tricalcium phosphate, a small amount of very dilute acetic acid is used to dissolve the calcium salt and the solution is then dialyzed; cholesterin precipitates are dried over concentrated H_2SO_4 and freed from cholesterin by extraction with ether. Various enzymes are adsorbed by basic hydrates, such as aluminium hydroxide and iron hydroxide; several others by different colloids,[297] kaolin, charcoal, infusorial earth,[1020] proteins,[873,875] freshly prepared precipitates of barium sulfate or magnesium carbonate, fatty acids, fibrin flakes, etc. Some enzymes, particularly trypsin, are carried down with the nucleoproteins. Trypsin can be precipitated by adding one drop of a saturated solution of safranin to 5 to 10 cc. of a faintly alkaline 0.5 per cent solution of the enzyme.[1026a] Cellulose filter paper will adsorb an impure enzyme-protein compound;[1303a] if rapidly dried in warm air the colloidal protein adheres to the paper and the enzyme may be easily dissolved out by placing the paper in water for fifteen to twenty minutes. A clear filtrate is thus obtained which contains the enzyme. Further details in regard to the purification of enzymes will be given later when the individual enzymes are discussed.

b. *Filtration and dialysis.* Various methods of filtration are used for the separation and purification of enzymes. Ptyalin, rennet, pepsin and pancreatin can be removed from solution by filtration through paper;[725a] however, filtration through paper may cause the adsorption of some of the enzyme.[365] Ptyalin and purified *Asp. oryzae* diastase pass through a Berkfeld filter, rennet is retained even under high pressure, while pepsin and pancreatin are partially retained. According to one report[1231] gastric lipase will pass through a Reichel filter but this observation could not be confirmed.[1187] Filtration through a bougie is utilized for the separation of bacterial cells from the enzyme solution, but some of the enzyme may also be retained. The reaction of the medium has an important influence in this connection;[570] invertase, catalase, pepsin and emulsin pass through a Chamberland filter if the liquid is neutral to phenolphthalein (pH 8.3) but not if neutral only to methyl orange (pH 4.4). The failure of an acid enzyme solution to pass through a filter is probably due to the adsorption of the acid protein by the filter, for an acid enzyme solution can be filtered through a bougie which has first been used to filter egg albumin.

A colloidal gel filter can be utilized for the filtration of enzymes. To provide this filter with a solid support, the gel should be precipitated upon filter paper by immersing a piece of filter paper in a solution of collodium (in acetic acid) and then dipping the treated paper in water. To avoid the formation of bubbles, a special apparatus has been devised which produces a definite pressure for saturation; in this way filters of various degrees of permeability can be obtained.[111] A 1 per cent hemoglobin solution may be used for testing the permeability of these filters. Gelatin solutions (3 per cent) have also been employed for the preparation of filters. A thin gelatin layer may also be applied to Chamberland filters. A Chamberland filter will usually retain lipase, zymase, pepsin and trypsin and is permeable to maltase, stomach lipase, aldehydases and malt proteases; a parchment filter will retain pepsin and peroxidase, and is permeable to invertase, diastase, emulsin, trypsin and pepsin; a collodium membrane retains pepsin and invertase and allows emulsin, lactase and trypsin to pass through.[404]

Dialysis is a process of diffusion, in which the degree of diffusion is determined by the number and size of the pores in the dialyzing membrane. It is used to separate substances consisting of large molecules from those consisting of smaller ones, as exemplified in the separation of colloids from crystalloids. The results of dialysis depend upon the nature of the membrane and on the type of the dialyzable solution. Enzymes do not pass through the dialyzer as readily in the presence of proteins as in their absence. Parchment membranes, pig and fish bladders, and collodium sacks[442,779] are chiefly employed for dialyzing purposes; different membranes will retain different enzymes. In some instances silk impregnated with cholesterin or lecithin, as well as other artificially prepared membranes, is also used.[1298] The enzyme solution is placed in the dialyzer, which has first been properly tested for permeability. The dialyzer is then placed in distilled water which is frequently renewed or in running water, if there is a large amount of inorganic salts in the enzyme preparation. An efficient apparatus for this purpose has recently been described.[1248] Enzyme preparations may also be purified by electrodialysis through a collodium membrane with a current of 22 to 220 volts and 0.0045 to 0.006 amperes.[462] The reaction of the medium is an important factor in dialysis.

Dialysis is often but one step in the purification of an enzyme;[1275] to obtain as pure an enzyme as possible, several of the above described processes may be necessary. For example, very pure invertase has been prepared by: (1) autolysis of yeast; (2) precipitation of the autolyzed liquor with alcohol; (3) adsorption by aluminium hydroxide, subsequent solution and dialysis; (4) adsorption with kaolin, subsequent solution and dialysis and (5) repetition of the third process.[633a]

4. MEASUREMENT OF ENZYME ACTIVITY. Enzymatic activity may be demonstrated either qualitatively or quantitatively. The formation of reaction products, the disappearance of the substrate or changes in the appearance and physico-chemical condition (viscosity, reaction, etc.) of the substrate form the basis for most of the methods. Several methods may be utilized in the study of one enzyme. For example, with diastase, the formation of sugar, the disappearance of solid starch and changes in the viscosity of starch solutions may all be measured. Quantitative results obtained by each of these three methods, however, are not identical, for the reactions depend upon dissimilar phenomena. Enzymatic material is often a mixture of different enzymes and one enzyme may use as a substrate the products formed by the action of another enzyme. Substances which contain diastase, for example, usually also contain maltase so that, in reactions upon starch substrates, not only is maltose formed which is the final product of pure diastase, but also dextrose, as a result of the action of the maltase upon the maltose. This fact together with the various side reactions are important sources of error in measuring enzymatic reactions. The activity of enzymes is influenced by various paralyzing and stimulating substances which may be present or added to the enzyme system, as well as by temperature, reaction and other factors. The enzyme solution to be tested should be freshly prepared or well preserved, since reactions, which are ascribed to enzymes, are often actually due to the action of contaminating bacteria. The latter may also destroy some of the enzyme. If the period of incubation is longer than 60 minutes, a disinfectant should be added and an incubator

may be employed, but for short periods of incubation a constant temperature water bath should be used. Parallel determinations should always be made with boiled enzyme as a control.

The units of measurement are relative and are different for each enzyme, method and investigator. Usually a definite amount of substrate changed or a definite quantity of products formed, in a definite length of time, is taken as a unit. In the case of invertase, for example, the inversion capacity of microörganisms has been defined as the figure which is obtained by dividing the amount of sugar inverted by the number of cells (with yeasts or bacteria) or by the grams of the dry weight (with fungi).[406] The inversion capacity of a substance may also be expressed by the ratio: $\frac{K \times \text{grams of sucrose}}{\text{grams of preparation}}$, K being the inversion constant.[1282] An enzymatic value for diastase may be obtained by dividing a definite weight of starch by the amount of enzyme required to digest it;[1120] one method consists in determining the smallest amount of diastase which so completely digests 250 cc. of starch paste (equivalent to 2.5 grams of anhydrous potato starch) in thirty minutes at 40°C. that 0.25 cc. of the digestion mixture will not produce any color change in 5 cc. of a diluted solution of iodine. A unit of trypsin may be defined as the smallest amount which requires 60 minutes to produce a 20 per cent change in the viscosity of a standard gelatin solution in one hour at 34°C.[900] A similar definition of an enzyme unit may be applied to the changes in the viscosity of starch solutions produced by amylase.[304]

CHAPTER IX

ENZYMES ACTING UPON FATS AND ESTERS (ESTERASES)

1. LIPASE (STEAPSIN). True lipases convert neutral fats to glycerin and fatty acids, according to the general formula:

$$R \cdot COO \cdot R^1 + H_2O \rightleftarrows R \cdot COOH + R^1 \cdot OH$$

until a definite equilibrium is established. In view of the fact that the lipase of certain organs, such as the stomach, decomposes not only true fats, but also lipoids and various esters, the whole group are often classified as *esterases.* The esterases are often divided into (*a*) animal and (*b*) plant esterases, on the basis of their origin;[276] the plant esterases can be further subdivided into: (1) *lipases* which act upon true fats and oils, and (2) *butyrases* which split the esters of lower fatty acids and mono- or polyvalent alcohols. In addition, there is probably a definite difference between lipases and esterases on the one hand, and the enzymes which act upon the esters of higher alcohols (cholesteryl-palmitate, etc.) on the other.[981] Lipases of different origin, such as animal and plant lipases, differ in their properties, and even between the lipases of the stomach, pancreas and liver there are certain differences, especially in their optimal reactions and ability to attack various esters. The optimum for gastric lipase lies between pH 4.0 and 5.0, those for the lipases of the pancreas, intestine, blood serum and woman's milk are between pH 7.0 and 8.6,[60,298] while bacterial lipase has its optimum at pH 7.2 to 9.0.[908] Castor oil lipase digests true fats actively, while liver and pancreas extracts attack lower esters more energetically.[42] Serum lipase (tributyrinase) resembles the lipases which digest neutral fats and ester-like lipoids.[425] Different fats are not attacked at the same rate by the Ricinus lipase.[275] Two different ester-hydrolyzing enzymes are probably present in various seeds as well as in animal organs, one a true lipase acting upon fats and the other an esterase acting upon lower esters, the first being more soluble in 1.5 N sodium chloride and the second more soluble in water.[419] The lipases obtained from the same organ in different animals, as, for example, pancreatic lipases, are usually similar;[1287,1288] any differences observed in their behavior can be explained by the effect of accompanying substances. A detailed study of the occurrence and importance of lipase may be obtained elsewhere.[923]

Preparation. To obtain lipase from castor oil beans they are allowed to germinate; when the radicles have protruded slightly, the endosperms are ground up in a mortar with 5 per cent sodium chloride. The solution is then filtered off and dialyzed. The enzyme may be precipitated by an excess of alcohol, washed and then dried. Ammonium sulfate may also be used for the precipitation of the enzyme. The castor oil seeds can be macerated directly in water (5 gm. to 60 cc.) to which 2 per cent acetic acid and 1 per cent chloral hydrate have been added. After twenty-four hours, approximately 58 per cent of the fat will be hydrolyzed. Another method is to grind up the oil seeds in water, and then to centrifuge out the debris. If the supernatant emulsion is incubated at 24°C., a scum containing the enzyme rises to the surface and is separated from the aqueous layer. This scum can then be used as a source of lipase by allowing it to act upon fat in the presence of water and a small amount of manganese sulfate.[580] This Ricinus lipase is an insoluble enzyme and can only be purified mechanically or by dissolving out the impurities. In all processes of lipase purification the enzyme must be left in contact with fat; it can be employed as an emulsion of fat and water.[1284] An active emulsion of the enzyme can be obtained by a rapid separation in a centrifuge at high speed of the non-acidified emulsion of freshly prepared castor oil beans. The enzyme can be concentrated by adsorption with infusorial earth, drying the paste in a desiccator, then extracting the oil with organic solvents; or by passing a stream of warm air over a thin layer of the emulsion. An esterase can also be prepared from soy beans, by extracting the ground beans with ether, then drying, grinding and sieving.[419]

Gastric juice lipase can be obtained by the method of Pavlov[953] or by extracting the stomach membrane with glycerin. Pancreatic juice is also an excellent source of animal lipase. This may be obtained through a fistula in a dog[743] or by a duodenal tube. Lipase can be prepared from blood serum by precipitation with ammonium sulfate. On shaking a lipase preparation with water and ether, the enzyme will be extracted in the water, but not in the ether. However, the lipase is soluble in ether in which an ester is dissolved. Fat-free lipase preparations are very sensitive to moisture and are rapidly destroyed. Lipase can be extracted from the pancreas and other organs with 1 to 3 volumes of 90 per cent glycerin containing 0.1 per cent sodium carbonate.[640] An active lipase has been prepared by chopping up fresh hog pancreases, previously freed from fat and membranous tissue, covering the mass with two volumes of alcohol for one day and then pressing out the fluid.[103] This turbid liquid is then precipitated with ether and the scum collected, washed in ether and dried. Lipase may also be obtained from swine pancreas by drying with acetone-ether and then extracting with 87 per cent glycerin. After centrifugalization, 5 volumes of water are added to the supernatant fluid; the resulting precipitate contains part of the lipase, most of the diastase and half of the trypsin. Adsorption of this precipitate with aluminium hydroxide or kaolin in weak acetic acid solution separates the lipase from the other enzymes. The lipase can be washed out with an ammonium phosphate solution (57 parts of 1 per cent $(NH_4)_2HPO_4$, 3 parts of NH_4OH and 40 parts of glycerin). The solution is then diluted with water and the phosphate precipitated with magnesia mixture. The lipase may be further purified by adsorption with kaolin and washing out, followed by adsorption with triesterin or cholesterin and washing out; the enzyme is then dried in vacuo, dissolved in benzol, centrifuged and washed with benzol, alcohol and ether. The lipase produces a clear solution in water and in glycerin and is more stable in the latter (liver esterase is much more stable). This purified preparation contains much ash and 10 per cent nitrogen but the protein tests are negative.

The *qualitative* demonstration of the presence of lipase is based upon the fact that the action of the enzyme-containing substance upon a neutral fat produces an increase in the content of the fatty acids. This increase is proportional to the strength of the enzyme. If two equal portions of enzyme solution, one of which has been boiled, are added to the same quantity of fat emulsion or milk, and the mixtures incubated at 34°C. for two or more hours, the acid concentration of the mixture containing the unboiled enzyme will be increased. This change in acidity can be demonstrated by the addition of an indicator such as litmus or brom-cresol-purple. Lipase can be tested qualitatively by dropping the enzyme solution (duodenal juice, etc.) on plates containing 40 parts of 2 per cent agar, 40 parts of 5 per cent starch and 1 part of any fat emulsion.[234] After incubation for one hour at 37°C. (or 55°C. to exclude bacterial growth) a saturated solution of copper sulfate or copper acetate is dropped on the area containing the enzyme. After five to ten minutes, if fat saponification has occurred, a blue green color appears.

The *quantitative* methods of estimating the action of lipase usually involve either, (1) the titration, with a standard solution of alkali, of the fatty acids liberated from fats, or (2) the measurement of changes in the surface tension of the substrate. The optimal conditions for the activity of lipase are described on page 137.

Kanitz method.[640,1048] Commercial olive oil or castor oil to which phenolphthalein has been added is shaken with a few drops of N/10 NaOH until a pink color and a uniform emulsion are obtained. Two to five cubic centimeters of enzyme solution are added to each of two flasks containing 10 cc. of this emulsion and a small amount of toluol. The flasks are then incubated for ten to twenty-four hours. Two other flasks to which the same amount of boiled enzyme is added serve as controls. At the end of the incubation period, 50 cc. of 96 per cent acid-free alcohol, 5 cc. of acid-free ether and 3 drops of a 1 per cent alcoholic solution of phenolphthalein are added to each flask. The mixtures are then titrated with N/10 NaOH until a rose tint is obtained. The average of the two controls is subtracted from the average of the two flasks with the unboiled enzyme and the difference indicates the amount of fatty acids liberated by the lipase. The actual amount of free fatty acids produced, calculated as oleic acid, is obtained by multiplying the number of cubic centimeters of N/10 NaOH required by 28.16. This amount may be used as an index of the strength of the lipase.

The Volhard method[1231] involves the extraction of the free fatty acids with ether. Egg emulsion is employed as a substrate. After a definite period of incubation, a portion of the reacting mixture is extracted with ether containing a small amount of alcohol, and a measured amount of this ether extract is titrated with a standard alkali solution.

A solution of butter fat in acacia milk has been employed for measuring lipase activity;[947] 15 grams portions of powdered acacia are ground in a mortar with 20 to 25 cc. of distilled water, until the gum is hydrated. Twenty grams of melted butter are then added, 2 to 3 cc. at a time, followed by thorough grinding. The volume is gradually increased with 15 cc. of warm water and the resulting emulsion then diluted with distilled water, with continued grinding, until a volume of 500 cc. is obtained. The final product contains

4 per cent fat; its reaction is approximately pH 5.0. Seventy-five cubic centimeter portions of this emulsion are placed in 200 cc. Erlenmeyer flasks, the proper amount of lipase (usually 1 cc.) added and the flasks incubated twenty-four to forty-eight hours. The acidity of the mixture is determined before and after incubation by withdrawing 25 cc. portions with a pipette and mixing them with 100 cc. of a solution containing 3 parts of acetone and 1 part of ether. This mixture is titrated to a faint pink color with N/10 alcoholic KOH solution, using 5 drops of a 1 per cent alcoholic phenolphthalein solution as an indicator.

Takata method.[1187] An emulsion of fat globules of small but uniform size prepared from milk, by repeated washing and centrifuging, is used as a substrate. The number of fat globules in 1 cc. of emulsion is about 8×10^7; the size of the individual fat globules is 3 to 3.5μ, as ascertained by direct micrometer measurements as well as by determinations by the Siedentopf-Zsigmondy method of estimating the size of colloid particles. Five to 20 cc. of the emulsion, 1 to 3 cc. of gastric juice (or any fluid suspected of containing lipase) and a small amount of thymol or toluol are placed in each of several flasks which are then incubated at 38°C. from one to forty-eight hours. A duplicate flask containing boiled enzyme is incubated as a control. At intervals during the incubation, 15 cc. of neutral alcohol and 15 cc. of ether are poured into one of the flasks and thoroughly mixed. This mixture is then titrated to neutrality with N/10 NaOH using phenolphthalein as an indicator. The difference between the amount of N/10 alkali required for the neutralization of each mixture and the amount necessary to neutralize the control indicates the quantity of fatty acids liberated by the lipase.

In addition to the direct titration of the acids liberated as a result of lipase action, the glycerin formed may also be determined.[963] The stalagmometric method based upon changes in surface tension cannot be applied to the determination of the hydrolysis of true fats but only to that of the lower esters. The activity of lipase may also be measured quantitatively by incubating a mixture of fat, sodium bicarbonate and lipase and then titrating the amount of CO_2 liberated from the $NaHCO_3$ as a result of the action of the acid formed.[1042]

One of the units suggested as a measure of lipolytic activity is the phyto-lipase unit, or the quantity of enzyme solution which will hydrolyze, in twenty minutes, 7.5 per cent of 2.5 grams of olive oil (of a saponification number 185.5) at pH 4.7 (obtained by adding 2 cc. of 0.5 N acetate buffer).[1284] A unit of lipase has also been defined as that amount of enzyme which will hydrolyze, in one hour at 30°C., 24 per cent of the olive oil in the following mixture: 13 cc. of ammonia-ammonium chloride buffer solution at pH 8.9 containing 10 mgm. of calcium chloride, 15 mgm. of albumin and 2.5 grams of olive oil.[1288] The lipase value of any pancreatic preparation is equal to the number of these lipase units in 10 mgm. of the particular preparation used.

The optimal reaction for gastric lipase apparently changes on purification, due to the fact that it is greatly influenced by the accompanying impurities.[535,1288] Preparations of human and canine gastric lipase which normally have their optima at an acid reaction can be so changed by adsorption and precipitation that they become more active in an alkaline medium. The optimum for gastric lipase is constant for each animal species, lying between pH 5.5 and 6.3, for carnivora, hares, and rabbits, and between pH 7.0 and 8.0 in case of other rodents, moles, horses and pigs.[535]

The synthesis of fats by means of enzymes can be measured by determining the decrease in the acid concentration which results from the combination of glycerin with fatty acids. To study the synthesis, 1 part of fatty acid, 3 parts of glycerin, an enzyme solution and a small amount of thymol are mixed and then incubated. Immediately before and after various periods of incubation, 5 cc. of the mixture are added to 20 cc. of 95 per cent alcohol and titrated with N/10 NaOH.[923,1298] A diminution in the amount of N/10 NaOH used indicates an increase in the synthetic process.

2. Butyrases and Other Esterases. Esterases can hydrolyze esters of the lower fatty acids of the ethyl butyrate type as well as aromatic esters. They are present in most animal organs, such as pancreas, stomach, liver, lung, etc., and in various body fluids, such as blood, milk, urine, intestinal and ascitic fluids, in various plant seeds and in different microörganisms. The hydrolysis of monobutyrin or other esters by the enzyme results in the formation of glycerin and butyric acid or the corresponding alcohol and fatty acid. This process can be readily studied, and the activity of the enzyme determined by titration with a standard alkali solution.

The body fluids and tissues may be used as sources of esterase or the enzyme may be prepared by any of the methods previously outlined for lipase (see p. 138). An active esterase can be obtained by grinding 100 grams of swine liver with sand and water, pressing the mass through cloth and making up the volume to 1000 cc. with distilled water.[959] The emulsion is allowed to stand for twenty-four hours, at 37°C. in the presence of toluol. After filtration, the liquid is kept for a few weeks at room temperature, and is then filtered clear. On dialyzing in collodium sacks and filtering, 90 per cent of the solid matter is taken out while the enzyme loses only 20 per cent of its strength. The solution is next half saturated with ammonium sulfate, and the inactive precipitate filtered off. The filtrate is then completely saturated with $(NH_4)_2SO_4$; the resulting precipitate which contains the enzyme is dissolved in water and then dialyzed, until free from sulfate. This solution is very active.

A purified liver esterase preparation may be made by extracting liver with dilute ammonia, evaporating the extract, then redissolving the crude product in a solution of acetic acid and ammonium acetate at pH 4.2.[1287] The enzyme is adsorbed, from the solution, by kieselguhr or aluminium hydroxide from which it is subsequently recovered with dilute ammonia. Such preparations are about twenty times as active as crude desiccated liver. The preparation loses about one-fifth of its activity during the first few days and then remains constant in strength. Liver esterase is distinct from pancreatic lipase in its behavior towards the isomers of certain optically active esters and in its susceptibility to the effect of certain substances. Sodium oleate, for example, will accelerate the hydrolysis of tributyrin by pancreatic lipase and will retard by 50 per cent the action of liver esterase.

The strength of esterase preparations is most easily measured by allowing them to act for twenty-four hours at 37°C. upon a 1 per cent aqueous solution of ethyl butyrate neutralized with sodium hydroxide. The amount of N/20 NaOH required to neutralize the butyric acid formed during incubation is used as an index of the strength of the enzyme.[419,420,643]

A simpler method has been based upon the changes in surface tension of an ethyl butyrate solution which result from enzyme action.[298,1032] These changes are measured by the number of drops of the enzyme-substrate mixture which will issue from a standardized 3 cc. pipette; the number of drops decreases with an increase in surface tension.

Changes in surface tension may also be determined by a Traube stalagmometer or a du Noüy tensionmeter.[354] The substrate is prepared by mixing 1 part of normal primary phosphate solution with 7 to 9 parts of normal secondary phosphate. Fifty cubic centimeters of saturated ethyl butyrate solution (prepared by shaking a few drops of ethyl butyrate in 1000 cc. of distilled water and then filtering), 2 cc. of the above phosphate mixture and 1 to 2 cc. of enzyme solution are thoroughly mixed and then incubated. By means of a dropping pipette or stalagmometer, the surface tension of the mixture is determined immediately before incubation and at intervals of thirty minutes for the next two or more hours. The hydrolysis of the ethyl butyrate results in an increase in surface tension which causes a decrease in the number of drops issuing from the pipette or stalagmometer. The substrate may be more simply prepared by shaking for 15 minutes 0.5 cc. of mono- or tributyrin with 100 cc. of a phosphate mixture at pH 8.0 (2 cc. of $\text{M}/10\ NaH_2PO_4$ and 98 cc. of $\text{M}/10\ Na_2HPO_4$) and then filtering through filter paper.

Another satisfactory and simple method consists in preparing and incubating the following mixture: 5 cc. 1 per cent glycerol triacetate (freshly prepared), 5 cc. phosphate buffer solution,[291] 0.5 cc. 0.3 per cent sodium glycocholate, 0.3 cc. pancreatic juice (or other enzyme-containing material) and 8 drops of 0.02 per cent phenol red or of another suitable indicator. The time taken to produce a definite change in the hydrogen ion concentration of the mixture, as shown by the indicator, is used as a measure of the lipoclastic activity. The shift in the reaction is usually traced only as far as pH 7.0, further change being considered too remote from the optimal pH. Average samples of pancreatic juice change the reaction from pH 7.8 to pH 7.0 within an hour; under these conditions about 25 per cent of the available glycerol triacetate is hydrolyzed. Control tubes containing the same mixture except for the pancreatic juice undergo only a negligible amount of hydrolysis.

3. Lecithinase. Lecithinase is present wherever lipase is found, i.e., in the stomach, liver, nerves, duodenal secretion, pancreatic juice,[1197,1298] hydrocele and ascitic fluids, various seeds, especially in castor oil beans, and in microörganisms, particularly in yeasts and *Asp. oryzae*.[22] Lecithinase acts best at a slightly alkaline reaction and is usually more thermostable than lipase.[440] While stomach and Ricinus lipase act upon lecithin, blood lipase does not.[1107] There

is a very marked parallelism between hemolysis and the hydrolysis of lecithin;[879] it has even been suggested that hemolysis is a result of the hydrolysis of the membrane lipoids of red blood cells.

Just as in the case of lipase, the qualitative and quantitative methods for demonstrating lecithinase are based upon the formation of acid from the substrate. The latter is usually a 2 to 3 per cent emulsion of lecithin which is prepared by dissolving 2 gm. of lecithin in 5 cc. of methyl alcohol by warming on a water bath;[1298] 100 cc. of distilled water are added to this constantly stirred solution, and an homogenous milky emulsion is formed. On warming this emulsion for a few minutes on a boiling water bath, the alcohol is evaporated off. The emulsion can be preserved for a long time. One to 5 cc. portions of enzyme solution are placed in each of four flasks, two of which are heated to destroy the enzyme. Ten cubic centimeter portions of the lecithin emulsion are placed in the four flasks, together with some toluol. The flasks are then stoppered and placed in the incubator for twenty-four hours. At the end of the period of incubation, 30 cc. of alcohol and a few drops of phenolphthalein are added to the contents of each flask which are then titrated with N/10 NaOH until a pink color is obtained. By subtracting the average for the control flasks from that of the flasks containing the unheated enzyme, a measure of the activity of the enzyme is obtained.

4. Cholesterinase. Cholesterinase converts cholesterin-ester into cholesterin. It has been definitely demonstrated in the liver of cattle and horses[678,1104] and in the spleen, nerves and blood.[286] There is a decided difference between the action of lipase or esterase and that of cholesterinase,[981] although the two enzymes may occur together in various animal organs. To study the activity of cholesterinase, the organ under investigation is thoroughly ground and divided into two equal portions. The free cholesterin and its ester are immediately determined in one portion, while the other is diluted with double its volume of water containing 1 per cent sodium fluoride and some thymol, and incubated for forty-eight hours. An increase in the free cholesterin is used as a measure of the action of the enzyme. Experiments should be made to determine whether adrenal cortex contains cholesterinase for if the enzyme can be demonstrated in this location, it would tend to prove that cholesterin is manufactured there and not simply deposited, a point which is repeatedly discussed.[500a]

5. CHLOROPHYLLASE. There has been described[1273] an enzyme which is capable of hydrolyzing (or rather alcoholyzing) chlorophyll into the alcohol, phytol, and a chlorophyllid:

$$\mathrm{R\ COO\ C_{20}\ H_{39} + C_2H_5OH = \underset{\text{ethyl chlorophyllid}}{R\ COOC_2H_5} + \underset{\text{phytol}}{C_{20}H_{39}OH}}$$

Chlorophyllase has been found in many plants, especially *Heracleum*, *Galeopsis*, *Stachys* and *Datura*. To demonstrate the action of the enzyme, fresh, undried leaf material is brought into contact with a freshly prepared chlorophyll extract. Ground leaves rapidly dried at a temperature below 40°C., in the absence of sunlight, can also be employed. Chlorophyllase is still active in highly concentrated alcohol solutions and in the presence of acetone. The hydrolysis of the chlorophyll can also be studied by a colorimetric method based upon the acid nature of the chlorophyllid. The latter can be extracted from ethereal solutions with potassium hydroxide, diluted with methyl alcohol and compared in a colorimeter with chlorophyll saponified and diluted with KOH in methyl alcohol.

6. PHOSPHATASES (ZYMO-PHOSPHATASES). Phosphatases are those enzymes which hydrolyze organic phosphorus compounds, namely esters of phosphoric acid and organic alcohols or alcohol derivatives; they can, therefore, be classified with the esterases. Among the most important of these esters, are the carbohydrate-phosphates, glycero-phosphates and phytin or inosite-phosphoric acid. Among the carbohydrate-phosphatases are those capable of hydrolyzing zymo-phosphate, or fructose-diphosphate, sugar-phosphate, especially saccharose-phosphate, starch-phosphate and nucleotides.

It has been demonstrated[519,597a] that yeast contains an enzyme which is capable of hydrolyzing fructose-diphosphate ester, according to the formula:

$$\mathrm{C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = C_6H_{12}O_6 + 2R_2HPO_4}$$

The action of the enzyme is measured by the formation of free phosphate. This enzyme has also been found in the intestinal membrane of swine and in the nervous tissues of horses,[396] as well as in higher plants, such as Ricinus seed, clover, oats and others.[393] The enzyme also acts synthetically upon a mixture of sugar and phosphate; this synthetic enzyme has been designated "*phosphathese.*" The optimal reaction is pH 6.5.

Saccharophosphatase which hydrolyzes saccharose-phosphate occurs in living yeast and in yeast juice.[880] It remains to be demonstrated whether this enzyme is different from zymo-phosphatase. The possibility of the existance of compounds of starch and organic phosphates and of their hydrolysis by specific phosphatases which are present in malt has been suggested.[19]

7. Glycerophosphatase. Glycerophosphatase converts glycerophosphoric acid, which is an important constituent of lecithin and other lipoids, into glycerin and phosphoric acid. This enzyme is present in the nerves, intestinal canal, faeces, to a certain extent in the lungs,[452,501] in castor oil beans, mustard seed, soy beans,[878] in yeast, clover[977] and in *Asp. oryzae*.[22] Glycerophosphatase which is water soluble may be obtained from Ricinus seed, by precipitating a dialyzed water extract with acetone.[420] The optimal temperature for the action of the enzyme is 35°C. and the optimal reaction is pH 5.4 to 6.0.[878]

A one per cent aqueous solution of sodium glycerophosphate is used for testing the enzyme; this salt is almost free from organic phosphates. The enzyme to be tested is added to 20 to 50 cc. of the sodium glycerophosphate solution and a small amount of toluol, and incubated for 24 hours. At the end of the period of incubation, the solution is freed from proteins by adding NaCl, kaolin and a few drops of concentrated acetic acid. The filtrate is then tested for free phosphate by the use of magnesia mixture. If the test is positive, the magnesium phosphate may be filtered off, the solution evaporated to dryness, ignited and the phosphate of the ash determined. This serves as a double check for the amount of hydrolysis that has taken place. The phosphate content of a control containing boiled enzyme should be subtracted.

8. Phytase. Phytase hydrolyzes phytin or the ester of inositephosphoric acid. This enzyme is present in rice, wheat and other plants,[1179] in certain fungi[342] as well as in the liver and blood of animals.[794] There is still some doubt, however, of the existence of this enzyme, since phytin readily decomposes with the formation of phosphoric acid.[613] Phytase is prepared by digesting finely ground wheat or rice bran with water at 2 to 3°C. and then precipitating the enzyme from the filtered solution with 95 per cent alcohol. Further purification is accomplished by drying the precipitate, extracting with water and reprecipitating. The activity of phytase is usually measured by determining the quantity of inorganic phos-

phoric acid or its salts which is produced by the hydrolysis of the phytin. The changes in the specific conductivity of a water extract of phytin and phytase afford a more convenient measure of the progress of the hydrolysis of phytin.[268] The optimal temperature is 55°C., although, during the first 15 minutes, hydrolysis proceeds more rapidly at 60°C. The optimal reaction is pH 5.4 to 5.5.[19]

9. Nucleotidase. Nucleotidase is an enzyme which is present in the pressed juice of liver and nerves, in blood and blood serum, in intestinal juice and membranes, and, to a more limited extent, in pancreatic extracts.[722] It is capable of hydrolyzing nucleotides, splitting off phosphoric acid and leaving behind the carbohydrate and purine or pyrimidine base:

Ribose-nucleotide → phosphoric acid + ribose-guanine. The decomposition of nucleic acids will be discussed in detail, under the nucleases (see pp. 212, 213).

10. Sulfatase. Sulfatase hydrolyzes ethereal salts of sulfuric acid, according to the reaction:

$$C_6H_5O \cdot SO_3K + H_2O = C_6H_5 \cdot OH + KHSO_4$$

Its action is measured by determining the diminution of organically combined sulfate or the increase in free phenol. It occurs in *Asp. oryzae* and in various organs (muscle, liver and nerves) of warm-blooded animals.[885a,887a]

CHAPTER X

ENZYMES ACTING UPON CARBOHYDRATES AND THEIR DERIVATIVES

1. ENZYMES ACTING UPON POLYSACCHARIDES. *a. Cellulase.* True celluloses, of the formula $(C_6H_{10}O_5)n$, are digested by a very limited group of living organisms which consists chiefly of fungi and bacteria. Although a large number of investigations have been made upon the decomposition of cellulose by anaerobic, aerobic, denitrifying and thermophilic bacteria, filamentous and higher wood-destroying fungi and actinomycetes, few deal with the formation and activities of the enzymes concerned in the process. Cellulose is hydrolyzed by the enzyme *cellulase*, with the formation of the disaccharide, cellobiose. The latter is split by the enzyme *cellobiase* into two molecules of glucose:

$$(C_6H_{10}O_5)_n + n\ H_2O = (C_{12}H_{22}O_{11})_n$$
$$C_{12}H_{22}O_{11} + H_2O = 2C_6H_{12}O_6$$

Thermophilic bacteria form these two enzymes;[991] by adding 50 cc. of an antiseptic, consisting of 2 per cent iodoform in acetone, to 2 liters of a culture of thermophilic bacteria, in which cellulose decomposition is taking place, the activities of the bacteria are stopped, but the enzyme action is not injured. The products of hydrolysis, cellobiose and glucose, then accumulate. By increasing the temperature of a culture to 67°C., bacterial growth is arrested and the action of cellobiase is repressed, but, due to the fact that cellulase is active between 20° and 70°C., cellobiose accumulates. Under normal conditions, the polysaccharides are decomposed completely to CO_2, water, butyric and other acids. Cellulase is said to be en endoenzyme and is insoluble in water; it is produced in response to the stimulus exerted by the cellulose upon the bacteria.[991] However, cellulase is secreted by the bacteria into the medium, for a clear zone is formed around each colony on a cellulose agar plate.[657]

Many of the cellulose decomposing enzymes, which have been isolated from fungi, are actually cytases acting upon hemicelluloses, rather than true cellulases. One of the fungi which has been named *Asp. cellulosae*, but which is more probably *Asp. fumigatus*, apparently produces cellulase.[376a] If this organism is grown in a liquid medium containing filter paper for three weeks, the liquid then separated from the paper and fungus growth, and filtered through a Berkfeld filter, the sterile filtrate will contain cellulase. If this is added to flasks containing sterile filter paper and incubated, in the presence of toluol, for twenty-eight days at 22°C., the paper will be macerated even though it has remained sterile. The cellulose-decomposing capacity of the digestive tract of various animals is no doubt due to the bacteria found there. However, certain invertebrate animals are capable of digesting cellulose by means of cellulase;[1314] the hepatopancreatic fluid of snails contains an enzyme which readily hydrolyzes cellulose to glucose.[642]

b. *Cytases* (*hemicellulases*). Chemically the hemicelluloses are not a sharply defined group but include various substances which, though they resemble celluloses, are more readily decomposed. They are hydrolyzed to glucose, mannose, galactose and pentoses. Cytases, the enzymes which digest this group are also capable of acting upon the mannans, galactans, pentosans, etc. The enzymes which digest these various substances are probably distinct. Cytase has been demonstrated in the endosperm of various plants, such as germinating barley,[200] lupines, palm seed,[889] other phaenerogams,[494a] in fungi,[657,1082] and bacteria. The formation of cytases also occurs in gastropods,[141] crustaceans and insects but not in higher animals. The enzyme is usually prepared by precipitating an aqueous extract of malt with alcohol, washing with absolute alcohol and drying in vacuo. The active powder contains both cytase and diastase. The optimal reaction for cytase is weakly acid. Most of the hemicellulases have not been studied sufficiently to be able to differentiate many of their specific properties.

Seminase converts mannogalactans to mannose and galactose. Its optimal temperature is 35° to 40°C. and its optimal reaction is weakly acid. It is formed abundantly by various plants, such as various leguminosae (*Medicago*, *Trigonella*), barley, rye and orchids.[172] The formation of reducing substances as a result of the action of seminase upon the mannogalactans serves both for the qualitative and quantitative demonstration of the enzyme. *Carubinase* is a closely related enzyme which digests carubin of the seed of *Ceratonia siliqua* and produces, as a result of hydrolysis, *d*-mannose and a small amount of galactose.[359]

Lichenase hydrolyzes the polysaccharide lichenin which is found in various lichens to cellobiose. This enzyme is found in the germinating seed of barley and other plants.[993] The gastric contents of oxen and pigs are said to contain lichenase,[642] but it is doubtful whether this enzyme occurs in the alimentary tract of any vertebrate, although it is present in the alimentary canal of each of many species of invertebrates, including arthropods, mollusks, echinoderms and annelids.[616] *Asp. oryzae* and *Asp. niger* also produce lichenase.[1072] *Xylanase* which digests xylans has been demonstrated in snails and in the larvae of coleoptera.[1114] *Gelase*, an enzyme which liquifies agar by hydrolyzing the carbohydrate, gelose, is present in *Bact. gelaticus*.[492] *Mannanase*, which produces laevodulin from mannans, and *laevodulinase*, which converts laevodulin into mannose, have been demonstrated in certain bacteria.[790]

c. Inulase (*inulinase*). Inulase converts the polysaccharide, inulin, into *d*-fructose. It occurs in the form of a zymogen, in the seeds of *Helianthus tuberosus*[494] and is also present in all monocotyledenous plants, in various fungi,[165a,305,1213] in yeasts,[729] bacteria,[55] in invertebrates and in the spleen and placenta of various vertebrates.[1189] This enzyme presents an interesting illustration of quantitative adaptation. Its action may be demonstrated by adding it to a solution of inulin and incubating. Due to the slow action of the enzyme, the incubation period should be one to three days. At the beginning and end of the incubation period, the enzyme-substrate mixture should be tested for reducing sugars using Fehling's solution, or by heating with resorcin and concentrated sulfuric acid and noting the presence or absence of a red color which is produced by free fructose. The increase in the amount of reduced copper serves as a quantitative index of the strength of the enzyme. The optimal temperature of the fungus enzyme is 55°C. and the optimal reaction is pH 3.8.[995]

d. Pectinase (*pectase*). This enzyme is capable of hydrolyzing pectins, which, according to Ehrlich,[368] are complex calcium-magnesium salts of an anhydro-arabino-galactose-methoxytetragalacturonic acid. This phenomenon is important in the maceration of vegetable tissues by parasitic fungi and bacteria. The enzyme is often considered to be a group consisting of three distinct enzymes: pectosinase, which converts pectose to pectin; pectase, which coagulates pectin, and pectinase, which hydrolyzes pectin to reducing sugars of the pentose series. Pectinase can also apparently convert pectin to pectic acid, methyl alcohol and acetone.[1207] *Pectase* is present in clover, alfalfa, apples, pears, potato juice, rape, beets, carrots and corn.[128] Active pectase may be prepared by allowing the pressed juice of clover, alfalfa or chopped potatoes to stand for 24 hours in the dark, in the presence of chloroform. The liquid is then filtered and precipitated with alcohol. The precipitate contains the enzyme. Coagulation of pectin solutions by pectase occurs in the presence of soluble salts of calcium, strontium and barium; the coagulum has been considered to be a pectate.[128] The action of pectase is often compared with that occurring in the coagulation of casein and fibrin, although the enzyme nature of pectin coagulation is occasionally doubted. The activity of pectase can be measured by determining the changes in the electrical conductivity of the solution or in its viscosity. The optimal reaction for pectase is pH 4.3.[400]

Pectinase is found in various plants, such as germinated barley,[172] in certain plant pathogenic and flax-retting bacteria, as for example *Bac. carotovorus*[628] and *Granulobacter pectinovorum* and in certain fungi such as *Rhizopus tritici* and *Fusarium cromophthoron.*[1141] The enzyme may be obtained by the precipitation of a culture of *Bac. carotovorus* with alcohol;[628] its optimal reaction is near neutrality. The formation of reducing sugars is used as an index of the action of the enzyme.

Pectinase can be prepared from fungi by the following method:[528] 500 grams of fruit or vegetables (turnips, sweet potatoes or carrots) are boiled in 1000 cc. of water and filtered. The filtrate and a small amount of ammonium nitrate, as a source of nitrogen, are placed in flasks and sterilized at a pressure of 15 pounds for fifteen minutes. *Rhizopus tritici* is then grown in this medium for two to three days. The presence of pectinase can be determined by dropping discs of raw sweet potato, 1 mm. in thickness, into the culture and incubating it at 45°C. The enzyme will macerate these discs. The maximal amount of pectinase is produced by the culture just before "fruiting" occurs. Either the liquid culture or the mycelium, after it has been ground with sand, can be used as a source of the enzyme. The latter is several times more active than the former.

e. Diastase (*amylase*). Diastase, or amylase, is the enzyme or group of enzymes which is capable of hydrolyzing starches and glycogen, as well as their primary and secondary decomposition products (soluble starch and various dextrins respectively) to maltose. The maltose is often converted into glucose during the hydrolysis of starch, as a result of the action of maltase which is frequently present in amylase preparations. Starches of different origin are not similarly attacked by the same diastatic enzyme.[1127] In addition to having different optimal temperatures and reactions, diastatic enzymes from different sources also differ in their action upon the same starch. Using $\frac{100 \times \text{grams of sugar formed}}{\text{grams of starch liquefied}}$ as a diastase unit, the following values have been obtained:[364]

SOURCE	DIASTASE UNITS	SOURCE	DIASTASE UNITS
Pancreas	1.01	Barley	2,400
Asp. oryzae	1.27	Oats	630
B. mesentericus	4.62	Rice bran	133
Malt	7.3	Leaves	11–12
Saliva	1.1	Chicory	11
Urine	0.83	Cress	10

Raw starch in the form of a paste is first converted by the action of diastase into soluble starch, then to dextrins and finally into maltose. This series of reactions, one following another, has led to various theories concerning the mode of action of this enzyme or group of enzymes. The starch-liquefying, or amylolytic action, has been differentiated from the sugar-producing, or saccharogenic action. Enzyme preparations differ in their capacity to liquefy starch and to convert starch to sugar, for example the diastase of *Asp. oryzae* has about six times the starch-liquefying power of malt diastase while the latter is six times as active in sugar production as the former.[1122,1240]

In view of the claim that starch is first hydrolyzed to dextrins and that the latter are then converted into sugar, processes which are not at all definitely established, it has been suggested that starch digestion is the result of the action of two enzymes, namely, *amylase* which liquefies starch and converts it to dextrin and *dextrinase* which forms maltose from the dextrin. These two diastatic enzymes, which are apparently present in malt, have also been described as *dextrinogenase* which hydrolyzes starch to dextrin and *saccharogenase* which converts starch or dextrin into maltose.[910] The first of these reactions, namely the liquefaction of starch by malt, proceeds 1.7 times as fast as the formation of sugar.[916] The two enzymes are differently affected by changes in reaction;[910] at pH 4.0, more of the dextrinogenase is destroyed than of the saccharogenase, so that it is possible to obtain a preparation practically free from the former. At pH 10.0, saccharogenase is destroyed more rapidly than dextrinogenase but the change at this point is not as definite. By heating the enzymes (at pH 6.0) at 70°C. for twenty minutes, saccharogenase is completely destroyed, while dextrinogenase is scarcely affected. They are also influenced differently by the concentration of sugar. The two enzymes are present in different amounts in different preparations. Some investigators differentiate between α- and β-amylases, on the basis of the mutarotation of the maltose formed;[996] the former has been described in barley extracts and is said to be destroyed when heated at 60°C. for twenty minutes; β-amylase is apparently formed only during the process of malting and is resistant to temperatures up to 80°C.[1180] However, other investigators do not consider it necessary to assume the presence in malt of two enzymes of different stability.[692] The terms, α-amylase and β-amylase, have also been applied to malt and animal amylases respectively.[692]

The nature of the substrate to be used in studying the action of diastatic enzymes is of prime importance. Commercial raw starch usually contains traces of plant proteins and about 15 per cent water. One to six per cent soluble starch, prepared by the method* of Lintner,[734] is usually employed for testing diastatic enzymes. Even soluble starch is not a pure chemical product for in it can be demonstrated two substances, "amylose" or "amylodextrin" (β-amylose of Meyer[818]) and "amylocellulose" or "amylopectin" (α-amylose of Meyer).[779b,818] The former produces a blue color with iodine and forms a paste with water. The latter forms a red violet color with iodine and settles out from water on cooling. Both substances are insoluble in cold water. Soluble starch usually consists of 17 per cent amylose and 83 per cent amylopectin;[1076] these two fractions may be separated by the centrifugalization of dispersions of commercial potato starch in water.[1127] Pancreatic and purified malt amylase, as well as the amylase of *Asp. oryzae*, digest β-amylose at a higher rate than they do α-amylose. According to one view,[996] the hydrolysis of starch by diastase proceeds in the stages illustrated in the following diagram:

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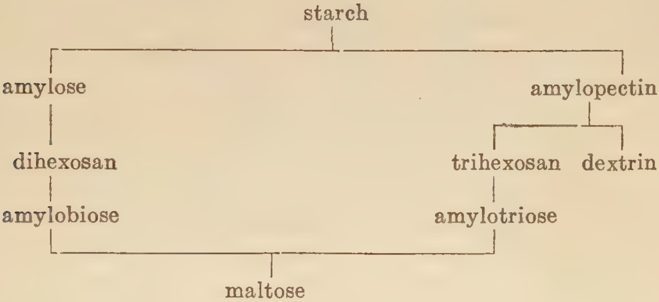
* *Preparation of soluble starch.* The Lintner soluble starch, which is frequently used in diastase determinations, is usually manufactured by the following method:[26] 500 gm. of purified potato starch are digested with 1000 cc. of dilute hydrochloric acid (sp. gr. 1.037) at room temperature (60° to 65°F.) for seven days, stirring the mixture daily. The mass is then washed very thoroughly by decantation, at first with tap water and later with distilled water, until the wash water is free from acid. The washed starch is collected on a filter paper placed in a Buchner funnel, pumped as dry as possible, and then spread out on a new unglazed plate. The starch should be dried at a gentle heat (110°F.) as quickly as possible. When dry the starch is triturated in a porcelain mortar and rubbed through a fine hair sieve.



A very pure starch preparation may be obtained by removing the gluten from wheat flour;[1004] the amylase of *Asp. oryzae* digests this pure starch much more rapidly than it does Lintner starch.[1005]

The elementary chemical formula of both starch and glycogen is $(C_6H_{10}O_5)_n$. Glycogen produces a red-brown color with iodine. Isolichenin is said to be identical with amylose and glycogen with amylopectin.[996] Raw starch is widely distributed in the plant kingdom, while glycogen is present in the muscles and liver of animals, and in fungi, yeasts and bacteria, but not in green plants.

Occurrence of diastatic enzymes. Enzymes capable of hydrolyzing starches and glycogen are widely distributed among animals, plants and microörganisms. The saliva of the great majority of animals contains diastase, which has been termed *ptyalin* by Berzelius.[131] Man, swine, rats and other animals secrete an active ptyalin. It is weak among herbivorous animals, such as horses, and may be entirely lacking in some carnivorous animals, as in dogs.[233] Of the various animal organs, the pancreas, especially that of pigs, is richest in diastase.[565] Diastase is also present in the liver, small intestine, muscles, nerves and other organs, as well as in the blood, urine, faeces, milk and cerebrospinal fluid. Diastatic enzymes are universally distributed in the plant kingdom, especially in the seeds of *gramineae* (barley, rye, wheat, corn, rice and oats). The enzymes become particularly active during the process of seed germination, especially in germinating barley or malt. Diastase also occurs in the leaves, stems, roots and branches of various plants. Diastase production is of common occurrence among the fungi, especially in certain *Aspergilli* (*Asp. oryzae*) and *Mucorales* (*Amylomyces rouxii*), among many bacteria, actinomycetes, algae and certain yeasts.

Preparation and purification of diastase. A large number of methods have been proposed for the preparation and purification of various diastatic enzymes. Different methods are required for enzymes from different sources. *Salivary* diastase or ptyalin can be prepared by precipitation with tri-calcium phosphate. For this purpose, calcium chloride and sodium phosphate are added to saliva. The resulting precipitate is washed with water and dissolved in dilute acetic acid, dialyzed and precipitated from the aqueous solution with alcohol.[263]

Pancreatic diastase may be obtained either from dried pancreas glands by extraction with water, glycerin or salt solutions, or from the pancreatic juice, by means of a permanent fistula or a duodenal tube. A purified pancreatic diastase can be prepared by the following method:[1121] 20 grams of pancreatin powder (dried and ground ox pancreas) are mixed with 200 cc. of 50 per cent alcohol, allowed to stand five to ten minutes at 15° to 20°C., and filtered. The filtrate is then added to 7 times its volume of a mixture consisting of 1 part of alcohol and 4 parts of ether and shaken with a rotary motion. An oily precipitate which contains the enzyme will settle in ten to fifteen minutes. This is separated from the rest of the liquid by decantation. The precipitated enzyme is then dissolved in a small amount of water at 10° to 15°C. and reprecipitated by pouring the solution into 5 times its volume of absolute alcohol. The flocculent precipitate is allowed to settle at 20°C., filtered and redissolved in 200 to 250 cc. of 50 per cent alcohol containing 5 grams of maltose. The solution is then placed in a 500 cc. collodium sack and dialyzed, at 15° to 20°C., against 2000 cc. of 50 per cent alcohol. The alcohol is renewed after fifteen hours and again eight or nine hours later. Dialysis is continued for a total period of forty-two hours. The enzyme in the collodium sack is then filtered and poured into an equal volume of alcohol-ether mixture (1:1). This mixture is filtered and then dried in a desiccator. Diastase rapidly loses its activity in distilled water, but less so in a solution containing NaCl and Na_2HPO_4.

Pancreatic diastase may also be purified by first adsorbing the lipase with aluminium hydroxide, and then removing the trypsin by adsorption with kaolin suspended in acetic acid.[1283] The diastase is finally adsorbed with $Al(OH)_3$ from a 50 per cent alcoholic solution of the preparation and washed with $(NH_4)_2HPO_4$ in $(NH_4)OH$. The diastase of liver and other organs can be extracted with glycerin. The organs themselves can also be used as sources of enzyme either when freshly macerated or after being dehydrated with alcohol and ether, dried and powdered.

Plant diastase is readily prepared from germinating cereal grains. Barley seed is soaked in water for ten to twelve hours, then spread in thin layers on trays and placed in a moist warm place to germinate. When the radicles are about $\frac{1}{4}$ inch in length, the grains are dried at a temperature not exceeding 40°C. and ground. The powder is mixed with four times its volume of water and allowed to stand for one to two hours with occasional shaking. The fluid is filtered and evaporated in vacuo to a small bulk. This preparation may be further concentrated by pouring it into alcohol, filtering off the precipitate which contains the diastase and washing it with alcohol. The diastase thus obtained may be redissolved and reprecipitated.

Malt diastase can be prepared by extracting one part of green or dry malt with two to four parts of 20 per cent alcohol for twenty-four hours.[734] This solution is filtered and precipitated with 2 to 2.5 parts of absolute alcohol, then with ether and dried in vacuo. The preparation may be redissolved in water, then reprecipitated and dialyzed. A highly active diastase is obtained by extracting germinated barley with 0.003 per cent acetic acid for at least six hours. Ammonium sulfate may also be used for precipitation.[927] Another method of preparation of malt diastase consists in extracting 5 kgm. of malt in 15 to 20 liters of water at 30°C. for eighteen hours, shaking the liquid every half hour. The liquid is then poured off and the residue washed with water and pressed. The combined liquid is filtered, placed in flasks with 500 grams of beer yeast and 500 grams of $CaCO_3$ and allowed to ferment for forty-eight hours at 25°C. After all the sugar has disappeared the liquid is filtered or centrifuged and then either precipitated with 2.5 times its volume of 95 per cent alcohol or concentrated in vacuo at 35°C. until calcium lactate begins to crystallize out. The enzyme is present in abundance both in the liquid and in the crystals. The latter can be separated by dialysis. The yeast employed may be first treated for twenty-four hours with 10 per cent sugar solution.[364,986] A very active preparation of malt diastase can also be prepared by electrodialysis and electro-osmosis.[462]

To obtain diastase from a growing plant, the following procedure may be used:[1147] *Phascolus* plants are cut off above the roots when they have reached a size of 7 to 10 cm. The plants are then crushed with sand in a mortar with sufficient water to make a thick mass. About 50 cc. of water are usually required for 50 plants weighing about 100 grams. A small amount of toluol is added and the mass is allowed to stand a few days so that the cells may autolyze and liberate their enzymes. The mass is then pressed through cloth and filtered. The filtrate is dialyzed twenty-four hours through a collodium membrane and is then filtered. About 100 cc. of juice are produced by this method.

Most purified diastase preparations have typical protein reactions. Purified pancreatic amylase has the following percentage composition: carbon 51.9, hydrogen 6.6, nitrogen 15.3, sulphur 1.0, phosphorus 0.8, and undetermined 24.4.[1121] Further information on the purification and chemical nature of malt diastase may be obtained elsewhere.[702,1122]

Fungus diastase is best prepared by means of *Asp. oryzae* which is used extensively in Japan for the preparation of Shoya sauce, the beverage saké, the soy bean product miso and other articles of food. The fungus is grown upon steamed bran or rice for forty to forty-eight hours. At the end of this time, the bran is overgrown with white mycelium. This mass is then extracted with water for fifteen to one hundred and twenty minutes. This water extract possesses strong diastatic properties and can be used as a source of enzyme. It can be preserved by the addition of toluol, chloroform, thymol, sodium fluoride or phenol, although the last has a destructive action upon the enzyme. It can also be concentrated in vacuo, at 30° to 40°C., and can then be kept without the addition of a preservative. The water extract is widely employed in the industries, and the concentrated form in food preparations. The enzyme may also be precipitated by adding the water extract to 2.5 times its volume of 95 per cent alcohol. The precipitate is then separated from the alcohol by centrifuging and filtering, washing with 95 per cent alcohol and drying rapidly. Under the names of Taka-diastase, Kashiwagi-diastase, and Digestin, this precipitated enzyme is extensively used in medicine, especially in Japan. A purified preparation can be obtained by extracting Taka-diastase with water, precipitating with ammonum sulfate, dialyzing and fractionally precipitating with alcohol.[1126] Diastase in dilute solutions can be fixed by raw commercial starch. The foreign substances can then be removed without the loss of the enzyme.[30]

Methods of measuring diastatic action. Qualitative tests for the presence of diastase may be made by adding 1 to 10 cc. portions of the enzyme to 10 cc. portions of 0.1 per cent starch solution and incubating the mixtures at 37° to 40°C. Small portions are removed at intervals and tested with an iodine solution to determine the disappearance of the starch, or with Fehling's solution to determine the increase in reducing substances.

Most of the available *quantitative* methods for measuring the diastatic action of enzyme preparations are modifications of three general processes which are based upon: (1) The disappearance of starch as indicated by iodine solutions; (2) the formation of sugar or other reducing substances in starch preparations, and (3) changes in the viscosity of starch solutions.

i. *The Wohlgemuth*[1295,1298] *iodine method.* A one per cent solution soluble starch (see p. 154) is prepared by shaking 1 part of soluble starch with 100 parts of cold distilled water, until the suspension is uniform. The mixture is then placed on a boiling water bath for ten to fifteen minutes or until solution is complete. Constant slow stirring is necessary to prevent sedimentation of the starch particles. The solution is then made up to the original volume with distilled water; it can be used immediately after cooling, or kept for a few days in a cold place, without the addition of an antiseptic. If sedimentation occurs on cooling, the solution should be filtered or decanted.

Varying concentrations of the enzyme solution are placed in a series of test tubes and 5 cc. portions of the 1 per cent starch solution are added, beginning with the tube containing the lowest enzyme concentration. One-half cubic centimeter of toluol is also placed in each tube. The tubes are then shaken, closed with stoppers and placed in a thermostat at 38° to 40°C. At the end of the period of incubation (usually twenty-four hours), the tubes are immediately placed in cold water and filled with cold tap water. One to 3 drops of N/10 iodine solution are added to each tube. The tubes are then shaken. The tube containing the highest dilution of enzyme, in which any blue red color (indicating the conversion of starch to dextrin) can be detected, is regarded as the limit of the activity of the enzyme. In case of doubt, more of the iodine solution should be added until a definite color is obtained. The strength of the enzyme can be expressed in terms of the number of cubic centimeters of 1 per cent starch solution which 1 cc., or 1 gram, of enzyme can convert to dextrin in twenty-four hours. This calculation is illustrated by the following example. Assuming the series of enzyme concentrations and the iodine reactions after twenty-four hours incubation at 40°C. to be as follows:

	ENZYME	IODINE REACTION
	cc.	
Tube 1	1.0	Yellow
Tube 2	0.5	Yellow
Tube 3	0.25	Yellow
Tube 4	0.125	Yellow
Tube 5	0.062	Yellow
Tube 6	0.031	Yellow
Tube 7	0.016	Red-yellow
Tube 8	0.008	Blue-red (limit)
Tube 9	0.004	Blue
Tube 10	0.002	Blue

Although the limit is tube no. 8, the next highest tube (no. 7) in which all the starch has disappeared, is usually taken as a basis for the calculations. Therefore, since 0.016 cc. of the enzyme solution are required to hydrolyze completely 5 cc. of 1 per cent soluble starch, 1 cc. of the enzyme could convert a proportionately larger amount of starch or:

$$0.016 : 5 = 1 : x \text{ and } x = \frac{5}{0.016} = 312.5$$

One cubic centimeter of enzyme solution then, could convert 312.5 cc. of 1 per cent starch to the dextrin stage, in twenty-four hours. The diastatic power may be expressed as $D^{40°C.}_{24\text{ hours}} = 312.5$. If a 2 per cent enzyme solution has been used, then the diastatic power of the enzyme would be $D^{40°C.}_{24\text{ hours}} = 312.5 \times \frac{100}{2} = 15{,}625$ per gram.

Wohlgemuth[1295] also suggested the use of a 0.1 per cent starch solution and N/50 iodine solution for a thirty-minute test. Two cubic centimeter portions of starch are added to the enzyme solutions in the series of tubes, which are immediately placed in a water bath at 38° to 40°C. At the end of thirty minutes, the iodine solution is added drop by drop to the tubes, without filling them with water. The calculations are carried out as above, on the basis of a 0.1 per cent starch solution. Salivary diastase (using undiluted saliva) can be tested by this short method. The same is true for various plant extracts. With pancreatic juice, liquid malt extract, or dilute *Asp. oryzae* enzyme, either 1 or 10 per cent enzyme concentrations are used, while the purified enzymes are tested in 0.1 per cent or lower concentrations of the enzyme. Several modifications of this iodine method have been described.[84,624,1120]

The diastatic value of human saliva has been found[1298] by this method to be: $D_{30\ \text{minutes}}^{38^\circ\text{C.}} = 62.5$ to 250 and $D_{24\ \text{hours}}^{38^\circ\text{C.}} = 1125$ to 5000. The diastatic power of human pancreatic juice is: $D_{1\ \text{hour}}^{38^\circ\text{C.}} = 625$ to 2500, $D_{24\ \text{hours}}^{38^\circ\text{C.}} = 12{,}000$ to 40,000. The starch-digesting activity of the pancreatic juice of the dog is quite similar: $D_{1\ \text{hour}}^{38^\circ\text{C.}} = 625$ to 2500, $D_{24\ \text{hours}}^{38^\circ\text{C.}} = 4{,}000$ to 40,000. The diastatic value of purified pancreatic amylase is about 2,000,000.[1122]

In titrating the diastatic power of the blood, the serum, diluted with normal saline, and a 0.1 per cent starch solution are employed. A twenty-four hour period of incubation is necessary because of the low diastase concentration in the blood. The following normal diastatic values have been reported:[1298]

Human serum	$D_{24\ \text{hours}}^{38^\circ\text{C.}}$	= 10 to 80
Dog serum	$D_{24\ \text{hours}}^{38^\circ\text{C.}}$	= 160 to 320
Rabbit serum	$D_{24\ \text{hours}}^{38^\circ\text{C.}}$	= 10 to 40
Normal human urine	$D_{30\ \text{minutes}}^{38^\circ\text{C.}}$	= 16 to 64
Human faeces	$D_{24\ \text{hours}}^{38^\circ\text{C.}}$	= 468.8

The variations in the diastase content of the blood of patients suffering from various diseases are described elsewhere (see pp. 73–75). The diastatic activity of malt may be determined by adding iodine to the enzyme-substrate mixture after incubation and titrating the excess of iodine with sodium hyposulfite.[1293] The amount of maltose produced during digestion can also be measured iodometrically with hyposulfite.[1274]

ii. *Methods based upon the determination of sugar produced.* Lintner's[734] method for measuring diastatic enzymes, based upon the determination of sugars capable of reducing Fehling's solution, is very commonly used. This method was originally suggested for malt diastase determinations, but various modifications have been utilized for measuring the diastase concentration of other preparations. This method has found extensive application in breweries. In principle, it is based upon the production of a definite quantity of maltose by the action of an extract of malt upon a definite amount of soluble starch and is performed as follows: 25 grams of powdered malt are extracted with 500 cc. of water for six hours at room temperature. The extract is then filtered and the clear filtrate used as the source of enzyme. One-tenth, 0.2, 0.3, ... 1.0 cc. portions of the malt extract are pipetted into ten numbered test tubes. Ten cubic centimeter portions of a 2 per cent solution of soluble starch are added to each tube. These are then kept at 21°C. for one hour. Five cubic centimeter portions of Fehling's solution are next added to each tube. They are then placed in boiling water (using a wire basket for holding the tubes) for ten minutes, and examined. The tube containing the smallest amount of enzyme in which the copper is all reduced is taken as the end point. The diastatic power (F) of malt is taken as 100, when 0.1 cc. of the malt extract produces just enough sugar, under the above conditions, to reduce completely 5 cc. of Fehling's solution. If the end point is produced by 0.25 cc., then $0.25:0.1 = 100:x$, or $x = 40$. If the original malt had only 60 per cent dry weight and 40 per cent moisture, then $F = \frac{40 \times 100}{60} = 66.7$. Since 0.2 grams of starch were present in each tube and the amount of maltose necessary to reduce 5 cc. of Fehling's solution is about 0.04 gram, the diastatic power as determined by the Lintner method is at a point at which only about 20 per cent of the starch is converted into maltose. The probable error of this method increases with the diastatic power of the enzyme tested.[1120]

Various modifications of the Lintner method have been suggested of which the following one is an example:[929] If the enzyme is in a solid form, it is first extracted for three hours at room temperature with sufficient distilled water to produce a 1 to 10 per cent solution.

Higher dilutions are employed with more potent enzymes. Ten cubic centimeters of the extract, or the diluted extract, are pipetted into a flask containing 100 cc. of a 2 per cent soluble starch solution which has previously been placed in a water bath at 40° to 50°C. The contents of the flask are then mixed, and the flask is kept in the bath for 30 minutes. At the end of this period, 10 cc. of 0.2 N sodium hydroxide are added to stop further enzymatic action. Graduated quantities of the digest are then poured into a series of test tubes, each containing 5 cc. of Fehling's solution. These tubes are next placed in a boiling water bath for ten minutes and shaken

TABLE XVI

Saccharogenic power scale for diastase from Aspergillus oryzae (40°C.)

OBTAINED VALUE	LINTNER SCALE *X Y*	OBTAINED VALUE	LINTNER SCALE *X Y*
cc.		*cc.*	
3.5*	735	10.5	126
3.75	643	11.0	120
4.0*	551	11.5	114
4.25	505	12.0	109
4.5	459	12.5	104
4.75	413	13.0	99
5.0*	367	13.5	94
5.25	336	14.0	89
5.5	305	14.5	84
5.75*	275	15.0	79
6.0	262	16.0*	69
6.25	249	17.0	65
6.5	236	18.0	61
6.75	223	19.0	57
7.0	210	20.0	53
7.25	197	22.0*	46
7.5*	184	24.0	42
7.75	178	26.0	38
8.0	172	28.0*	34
8.25	166	30.0	31
8.5	160	33.0	27
8.75	154	36.0*	24
9.0	148	37.0*	23
9.25	143	39.0	21
9.5*	138	45.0	19
9.75	135	50.0	17
10.0	132		

* Experimental value.

once during that period. The smallest quantity of the digested starch solution which just reduces the 5 cc. of Fehling's solution is determined. It is usually more convenient to use at first only 2 or 3 tubes containing widely different quantities of the digest in order to determine the approximate concentration of the digest to be used. The reduction of Fehling's solution is then carried out within narrow limits of this approximate value. Having thus determined the smallest amount of digested starch solution which will just reduce 5 cc. of Fehling's solution, the Lintner value can be calculated as is

TABLE XVII

Saccharogenic power scale for malt diastase (40°C.)

OBTAINED VALUE	LINTNER VALUE X Y	VALUE OBTAINED	LINTNER VALUE X Y
cc.		*cc.*	
3.5*	735	10.5*	92
3.75*	551	11.0	89
4.0*	367	11.5	86
4.25	336	12.0	83
4.5	305	12.5	80
4.75*	275	13.0	77
5.0	262	13.5	75
5.25	249	14.0	73
5.5	236	14.5	71
5.75*	223	15.0*	69
6.0	210	16.0	65
6.25	197	17.0	61
6.5*	184	18.0	57
6.75	177	19.0	53
7.0	170	20.0	49
7.25	163	21.0*	46
7.5	157	22.0*	44
7.75	151	24.0	40
8.0	144	26.0	36
8.25*	138	27.0	34
8.5	132	28.0	33
8.75	127	30.0	30
9.0	122	33.0	27
9.25	117	35.0*	23
9.5	112	36.0	22
9.75	107	39.0	20
10.0	102	45.0	18
		50.0	16

* Experimental value.

illustrated in the following example: 5 cc. of the digestion mixture (100 cc. of 2 per cent starch containing 10 cc. of a 2 per cent solution of the diastase of *Asp. oryzae*) are required to reduce 5 cc. of Fehling's solution. As shown in Table XVI, the Lintner scale xy for 5 cc. is 367. Y is the percentage of enzyme concentration and as the Lintner values (Tables XVI) are based upon a one per cent solution of enzyme, the Lintner value in this example will then be $\frac{367}{y}$ or $\frac{367}{2}$ or 183.5. Different tables are required for different enzymes and different temperatures of incubation. The Lintner values for malt diastase at 40°C. are illustrated in Table XVII.

A gravimetric method has been recommended for the measurement of pancreatic diastase;[1120] it is based upon the actual determination of the amount of sugar produced by the action of the enzyme upon starch. The enzyme is dissolved in pure water, if it is to be tested immediately, or in water containing 4 cc. of M/50 Na_2HPO_4 per 100 cc., if it is to be tested later (not more than an hour after dissolving the enzyme). Three hundred milligrams of NaCl and 7 cc. of M/50 Na_2HPO_4 per 100 cc. of the final reaction mixture are used as activating agents (for pancreatic diastase). Four 1 cc. portions of enzyme solution containing respectively 0.2, 0.5, 0.8 and 1 mgm. of the enzyme are placed in four 200 cc. Erlenmeyer flasks. One hundred cubic centimeters of a 2 per cent soluble starch solution previously warmed to 40°C. are added to each flask and digestion is carried out at 40°C. for thirty minutes. At the end of the incubation period, 50 cc. of Fehling's solution are added to each flask which is then immersed in a bath of boiling water. The precipitate is quickly filtered through Gooch crucibles with a thick felt of asbestos. The cuprous oxide is washed first with hot water, then twice with alcohol and ether, dried and weighed (the weight should not exceed 300 mgm.). A blank should be substracted. The diastatic power of a sample may be obtained by dividing the value of K (Table XVIII) corresponding to the weight of Cu_2O found, by the number of grams of enzyme used to effect this conversion. This is illustrated by the following example. It was found[1120] that the action of 0.0312 gram of enzyme upon a 2 per cent starch solution caused the precipitation of 100 mgm. of Cu_2O. The K in Table XVIII for 100 mgm. of Cu_2O is 31.2, therefore, the diastatic value would be $\frac{31.2}{0.0312}$ or 1000. (One hundred milligrams of Cu_2O

contains 89.4 mgm. of copper, the amount which would be precipitated by 82.0 mgm. of maltose.)

A change in the *polarization* of light can also be used for measuring diastatic action.[481] Four grams of soluble starch are dissolved in a mixture of 180 cc. of water, 10 cc. of N/10 acetic acid and 10 cc. of N/10 sodium acetate (pH 4.7). The diastase can be prepared by macerating 25 grams of ground distiller's malt with 500 cc. of water for one hour, stirring the mixture frequently and then filtering it. Two cubic centimeters of the diastase solution are added to 200 cc. of the 2 per cent starch solution and the mixture incubated in a water bath at 21°C. for one hour. At the end of this time, 2 cc. of strong ammonium hydroxide are added and, after waiting twenty-five minutes in order that the mutarotation may disappear, the degree of rotation is determined. The degree of rotation of a control 2 per cent starch solution without the diastase is subtracted.

TABLE XVIII

The action of pancreatic amylase upon a 2 per cent starch solution[1120]

Cu_2O	*K*	Cu_2O	*K*	Cu_2O	*K*
mgm.		*mgm.*		*mgm.*	
30	9.1	130	40.9	230	74.8
40	12.2	140	44.2	240	78.2
50	15.3	150	47.5	250	81.8
60	18.4	160	50.8	260	85.4
70	21.6	170	54.1	270	89.0
60	24.8	180	57.5	280	92.6
90	28.0	190	60.9	290	96.3
100	31.2	200	64.3	300	100.0
110	34.4	210	67.8		
120	37.6	220	71.3		

In addition to the above methods for measuring diastatic action, there should also be mentioned the *glycogen* method; it consists in the addition of an enzyme solution or organ suspension to a 5 per cent glycogen solution, containing a small amount of toluol. At various intervals, the amount of unchanged glycogen in the mixture is determined. The course of the reaction may also be studied by the use of a polariscope or by titration. The use of a substrate of 0.3 per cent glycogen solution and the measurement of its concentration, by a nephelometer, after various intervals of incubation, have also been recommended.[1040]

A simple and rapid method has been devised for the determination of blood diastase, which has the advantage of requiring only 0.4 cc. of blood and also of avoiding the sources of inaccuracy which are inherent in methods in which picric acid is used.[470]

iii. *Viscometric method.* The activity of preparations containing amylase may be determined quantitatively by measuring the changes in the viscosity of starch solutions.[304] The procedure consists essentially in the substitution of a boiled and autoclaved 2 to 6 per cent starch solution for the 3 per cent gelatin substrate used in the viscometric method for the determination of pepsin and trypsin[900] (see p. 210). A starch solution two and a half times as viscous as water is the most suitable for accurate measurements. With some preparations of Lintner starch, a 2 per cent solution is sufficient, while with others as much as 6 per cent is required. Two to 6 grams of Lintner starch are added to 100 cc. of distilled water; the mixture boiled one minute, then autoclaved at a pressure of 18 pounds for fifteen minutes and filtered through muslin. Very little hydrolysis occurs as a result of this treatment, but if starch is heated in very acid or alkaline solutions, caramelization is produced. The reaction of this solution is usually pH 5.6, but if other hydrogen ion concentrations are desired, a double strength starch solution is made and added, after cooling and filtering, to an equal amount of M/10 phosphate or other buffer mixture at the desired reaction. These starch solutions should be used within twenty-four hours after preparation.

Ten cubic centimeters of this starch solution are placed in each of two large Ostwald viscosimeters immersed in a glass water bath at 34°C. and the time required for 5 cc. of the solution to flow through the capillary tube is determined several times with a stop watch. This time value will hereinafter be designated as the "viscosity" of the solution. The viscosity of starch solutions prepared in this way is usually stable, but occasionally a preparation may be found which decreases in viscosity.[1056a] Such solutions are useless for amylase determinations. If the initial viscosity is stable, 0.1 to 0.4 cc. of the preparation containing amylase is added to one of the viscosimeters and the same amount of the enzyme preparation, after inactivation by boiling, to the other as a control. The contents are then thoroughly mixed by shaking and the time noted. The viscosity of these mixtures is then determined at intervals of three or more minutes for a period of one to two hours.

The results obtained may be plotted on coördinate paper, the abscissae representing the elapsed time between observations and the ordinates the observed viscosities expressed as percentages of the initial viscosity; the initial viscosity which is measured in seconds being recorded as 100 per cent. For example, if the observed initial viscosity of the starch solution before adding the enzyme is one hundred and seventeen seconds, it is represented on the ordinate scale as 100 (abscissa scale 0). If the observed viscosity, 12 minutes after adding the enzyme, is ninety-seven seconds, the ordinate would then be 83 (i.e., $117 : 100 = 97 : x$ and $x = 83$) and the abscissa 12. This apparently complicated, though really simple slide-rule method of recording results is necessitated by the fact that since several different viscosimeters are usually used, the initial viscosities are frequently not the same and, therefore, must be reduced to the same scale.

In studying the effect of trypsin upon the viscosity of 3 per cent gelatin, it has been found that unless very concentrated solutions of enzyme are used, the time required to effect a definite percentage of change in the initial viscosity of the substrate is inversely proportional to the amount of enzyme employed, that is double the amount of enzyme requires half the time to cause the same change (Arrhenius "Q × T" rule).[900] The same observations are true for the changes effected in the viscosity of starch solutions. On the other hand, the percentages of change produced in the initial viscosity of gelatin or starch solutions in a definite period of time are not proportional to the amounts of enzyme employed. It would, therefore, seem logical that the activity of an enzyme should be defined in terms of the time required to effect a definite change in its substrate rather than in terms of the amount of change produced in a definite time. It has been suggested that a unit of trypsin be defined as that amount of enzyme which requires sixty minutes to reduce the initial viscosity 20 per cent. It has been found that if 0.1 cc. of an enzyme preparation requires sixty minutes to reduce the initial viscosity 20 per cent, 0.2 cc. requires thirty minutes to reduce it a similar amount. If the initial viscosity is reduced 20 per cent in ten minutes, the enzyme preparation is assumed to contain $\frac{60}{10}$ or 6 units, etc. An amylase unit may be similarly defined. It has also been noted in a large series of amylase reactions that, except in those of great rapidity, the amount of enzyme which requires sixty minutes to reduce the initial viscosity 20 per cent, will reduce it 10 per cent in an average of 19.2 minutes and 5 per cent in 8.4 minutes. Therefore, if the reaction has been so slow that the initial viscosity has not been reduced 20 per cent during the period of observation, the number of units can be determined from the time required to produce a 10 or 5 per cent change.

The activity of *dextrinase* preparations may be similarly determined by using a 15 per cent dextrin solution as a substrate.[786]

The rate of the hydrolysis of starch by the amylase of *Asp. oryzae* is practically the same whether measured viscometrically, polariscopically or chemically.[786] The advantages of viscometric determinations for enzymatic activity are the ease and accuracy with which frequent observations of the enzyme-substrate mixture can be made. Only by measurements at short intervals can the fundamental effect of enzymes be studied, namely, the *rate* at which changes are produced in the substrate.

Another modification of the viscometric, or the amylo-liquefying method, is carried out as follows:[931] 7.5 grams (dry weight) of purified potato starch are made into a paste with 450 cc. of water. Two cubic centimeters of a buffer mixture consisting of equal parts of M/6 citric acid and M/6 Na_2HPO_4 and 2 cc. of the enzyme solution are added to 18 cc. of this starch paste and the mixture placed in a thermostat, at 40°C., for thirty minutes. Two cubic centimeters N/2 NaOH and 2 cc. of the above buffer mixture are then added and the viscosity of the mixture determined in a large Ostwald viscosimeter at 18°C. If the time required for the enzyme-substrate mixture to flow through the capillary tube of the viscosimeter is twenty seconds, the number of units of amylo-liquefying amylase in the preparation (*Asp. oryzae*) is regarded as 100; if the time required is twenty-four seconds, the number of units is 50; if thirty-two seconds, 25 units; if forty-seven seconds, 10 units and if over 80 seconds, no digestion has occurred. In Table XIX are illustrated the results of determinations of the activity of several diastase preparations made by this, Wohlgemuth's,[1298] and Lintner's[734] methods.

Influence of temperature and reaction. The optimal temperature for the action of diastase lies between 40° and 56°C., although the amylolytic action of certain enzymes such as that of *Asp. oryzae* and of malt is still present at 70° to 80°C. However, at the latter temperatures, the enzyme is rapidly destroyed if it is in an aqueous solution. When dry, the enzyme resists a temperature of 150°C. The diastatic enzymes of animals, microöganisms, grasses and germinated grain vary in their optimal temperatures, as well as in their optimal reactions and ability to transform starch. The optimal temperature for animal diastase is usually 38° to 40°C., for milk diastase 37°C.,[1260] while for germinated grain it is between 54° and 56°C.[264] The optimal as well as the destructive temperature is affected by the presence of extraneous matter.[253,288] The ability of an enzyme to withstand increased temperatures depends to a large extent upon its reaction,[388] ptyalin being most stable at pH 6.0 to 6.1. The concentration of sodium chloride greatly influences the thermostability of enzyme solutions, the greatest stability being present in a sodium chloride solution which is 10 times more concentrated than that in which the greatest activity occurs.[388] If the reaction is kept constant, the sensitivity of an enzyme to temperature increases with dilution. Pancreatic diastase has its optimum at 35°C. in a salt-poor solution and at 50°C. in the presence of 0.2 per cent NaCl; it is destroyed at 65° to 70°C.[1218] (The destructive temperature has been defined as that temperature at which the activity of the enzyme is reduced 50 per cent in one hour.[406]) At 60°C., malt amylase is destroyed in 1 hour; the amylase of other plants is even more sensitive, *phaseolus* amylase having its destructive temperature at 45°C. It has been reported[491] that amylase, especially that of *Asp. oryzae*, which has been inactivated at very

TABLE XIX

Comparison of amylo-liquefying, amylo-dextrinizing and amylo-saccharifying activity of several diastase preparations[932]

ENZYME PREPARATION	AMYLO-LIQUEFYING UNITS	AMYLO-DEXTRINIZING UNITS	AMYLO-SACCHARIFYING UNITS
Asp. oryzae (purified enzyme)	36,000	50,000	4,040
Germinated barley (purified enzyme)	20,000	33,340	6,100
Ungerminated soy beans (purified enzymes)	800	260	21,000
Asp. oryzae	1,000	833	206
Rhizopus delemar	50	67	11
Germinated barley	125	555	166
Germinated wheat	360		576
Germinated oats	160		41
Ungerminated rye	13	55	132
Ungerminated wheat	1.4		118
Ungerminated barley			0
Ungerminated oats			0

high temperatures (up to 110°C.) can be regenerated by means of starch or alkali but these observations could not be confirmed.[388]

The optimal reaction for diastatic enzymes depends on the nature of the buffer used. The optimum for salivary diastase, in a phosphate buffer solution, is pH 6.4 to 6.6, and, in an acetate mixture, pH 5.6. Amylopectinase has corresponding values of pH 7.1 and 5.5.[505] With unbuffered starch solutions the optimum for the starch-liquefying activity of the amylase of *Asp. oryzae* is pH 3.0, but with buffered starch solutions it is pH 4.0.[786] Pancreatic, malt and *Asp. oryzae* diastase have different reaction optima and minima for saccharogenic activity as shown in Figure 6.[1129] The optimal reaction for the saccharogenic action of malt amylase coincides with the isoelectric point or zone (pH 4.3 to 4.5) of the enzyme.[1134] Salivary diastase is most active at a weakly acid to neutral reaction (pH 6.4), pancreatic diastase in a weakly alkaline mixture (pH 8.0) and malt diastase at an acid reaction (pH 4.9 to 5.2).[136] In general, plant diastases are most active at pH 5.0 to 5.4.[1147] Diastase, like pepsin, is a negatively charged ion.[478]

Activators. The activity of diastatic enzymes is increased by the addition of various salts or inorganic activators. NaCl and KCNS have the greatest effect, KCl, KBr, NaBr, NH_4Cl, Ca-, Mg-, Sr- and Ba- chlorides are next, followed in order by nitrates, iodides and sulfates. The anions are more active than the cations.[136] Some enzymes, such as salivary diastase, are absolutely inactive in the total absence of salts.[406] The maximal accelerating effect of NaCl is in a concentration of 1.0 to 1.6 per cent.[388] The addition of neutral salts (NaCl, KCl), however, does not exert any activating effect upon the diastase of *Asp. oryzae* or malt if commercial soluble starch which contains salt is used as a substrate.[1126] The optimal concentration of NaCl for pancreatic amylase is 0.05 mol per liter if purified salt-free soluble starch is used as a substrate.[1129] Further information on the influence of acids and salts upon diastatic enzymes may be obtained elsewhere.[505, 923, 1124]

Among the organic activators are various amino acids, especially asparagine. Histidine, tryptophane and lysine do not increase the amylolytic action of diastase as markedly as the mono-amino acids.[1130, 1132] Lysine, however, has a favorable influence upon the saccharogenic action of the enzyme. Since diastase (pancreatic) is often regarded as a protein substance which gradually becomes inactivated through hydrolysis in the aqueous medium in which it acts, the apparent activating influence of certain amino acids has been explained as being due to the retardation of this hydrolysis of the enzyme or to a protection of the enzyme from deterioration (gradual hydrolytic destruction). According to this view, the lysine of the enzyme molecule is so bound that it is not split off until after the enzyme has lost its amylolytic activity so that the addition of lysine does not affect the amylolytic action. The saccharogenic activity, however, depends upon the presence of lysine. The addition of lysine to the enzyme solution, therefore, would tend to conserve the saccharogenic activity of the enzyme by diminishing the rate of its deterioration at this later stage. The addition of normal human serum to duodenal contents and to extracts of *Asp. oryzae* increases their starch-liquefying activity, although the serum itself has no starch-liquefying action.[907] Heating the serum to 56°C. partially destroys its accelerating effect. This is just the reverse of the influence of serum upon preparations of trypsin, and it has been suggested that in addition to antitrypsin, serum contains an amylase accelerator.[907]

Paralyzers. Fluorides, as well as the salts of heavy metals, especially those of mercury, copper[524,915,1029] and silver[852] have an injurious effect upon enzymes. Iodine and other halogens (Br, Cl) are also inhibitory, 1.6×10^{-5} N iodine solution reducing the activity of ptyalin 52 per cent in eleven minutes.[122,406] A 0.05 N formaldehyde solution will reduce the strength of salivary diastase 50 per cent in one hour. Malt amylase is also greatly injured by various agents, 1.39×10^{-7} N iodine reducing the activity of the enzyme 48 per cent in ten minutes, 9.03×10^{-4}N Na_2SO_4 64 per cent in ten hours, 2.14×10^{-4}N KCN 33 per cent in ten minutes and 3.0×10^{-3}N NaF 3 per cent in five hours.[916] The inhibitory effect of various chemicals upon the action of malt diastase decreases in the following order: mercuric chloride, uranyl sulfate, formaldehyde, mercurous chloride, mercurous nitrate, zinc sulfate and iron chloride. Hydrocyanic acid has no effect. Further information on the influence of antiseptics and other inorganic and organic substances upon the action of diastase may be obtained elsewhere.[252,953]

2. ENZYMES ACTING UPON TRISACCHARIDES. *a. Raffinase.* Raffinase converts raffinose into fructose and the disaccharide melibiose. The latter is further hydrolyzed by melibiase into dextrose and galactose. The second enzyme acts much more slowly than the first.[139] Raffinose can also be hydrolyzed into sucrose and galactose as a

result of the action of emulsin.[879a] Raffinase is produced in the intestine and stomach of mollusks, and also occurs in the digestive juices of crustaceans, insects and larvae. It is formed by top and bottom yeasts,[1277] by *Asp. niger* and *P. camemberti* especially when grown in a lactose medium. The action of raffinase may be determined either by means of a polariscope or by titration with Fehling's solution. The dextro-rotation of raffinose decreases as a result of the action of raffinase (due to the formation of levulose) and finally approaches zero, while the reduction of Fehling's solution gradually increases. The optimal reaction for raffinase is pH 4.5 to 5.0 at 30°C.[1277] This is very similar to that of invertase, but the former enzyme is less sensitive to a reaction of pH 2.0.

b. Gentianase. Gentianase hydrolyzes gentianose to fructose and the disaccharide gentiobiose.[139] The latter is further hydrolyzed by *gentiobiase* into two molecules of dextrose. This process can also be produced by a combination of invertase and emulsin. Gentianase has been demonstrated in the digestive juices of various mollusks and crustaceans,[139] as well as in certain fungi (*Asp. niger*) and top yeasts. Just as in the case of raffinase, the action of gentianase can be measured by a change in the polarization of light and an increase in the reduction of Fehling's solution.

c. Melezitase. Melezitase hydrolyzes the trisaccharide, melezitose, to dextrose and the disaccharide, turanose. The enzyme is formed by *Asp. niger*.[168]

d. Stachyase. Stachyase converts the tetrasaccharide stachyose, which consists of glucose, fructose and two molecules of galactose, into levulose and a mannosaccharide. The last is then hydrolyzed to dextrose and two molecules of galactose. Stachyase is found in the digestive juice of crustaceans and mollusks.[139] The course of hydrolysis can be measured by a change in the polarization of light and an increase in the reduction of Fehling's solution.

3. ENZYMES ACTING UPON DISACCHARIDES. The enzymes which hydrolyze simple compounds of sugars (hexoses) are often termed hexosidases,[406] and are divided into α-hexosidases and β-hexosidases, depending upon the structure and chemical configuration of the substrate. The α-hexosidases are divided into α-glucosidases, including α-methylglucosidase, maltase, and trehalase and α-fructosidases, including invertase. The β-hexosidases are differentiated into β-glucosidases, including emulsin, oxynitril-β-glucosidases (prunase, amygdalase), the glucosido-β-glucosidases

(cellobiase, gentiobiase and iso-maltase) and into β-galactosidases, including lactase and melibiase. These enzymes will be considered in the order of their importance, with particular stress upon invertase, maltase, lactase and emulsin. The first three act upon disaccharides and the last chiefly upon glucosides. A detailed study of these enzymes, especially in regard to their chemical structure may be obtained elsewhere.[406,923]

a. Invertase (*invertin, saccharase, sucrase*). Invertase converts sucrose or cane sugar into glucose and fructose according to the following reaction:

$$C_{12}H_{22}O_{11} + H_2O = C_6H_{12}O_6 + C_6H_{12}O_6$$

This process can also be produced by means of acids.

Occurrence. Invertase is well distributed in nature. It is found in the membranes of the small intestine, in the blood, pancreatic juice and pancreatic extracts of human beings and animals, the digestive tract of insects, including bees (honey invertase[876]), and in snails. It is well distributed in the plant kingdom[644] occurring in green leaves, fruits, grains,[934] stems, potato tubers, pollen and, to some extent, in roots, especially in the sugar beet.[1026] It is also found among various fungi, including certain Aspergilli, Penicillia and a few Mucorales, in several species of Actinomyces and bacteria[689] and is especially plentiful among yeasts. Invertase is found so abundantly in yeasts (except in the true wine yeasts) that these usually serve as a source of the enzyme.

Preparation and purification of invertase. A large number of methods are available for the preparation of this enzyme, chiefly from yeast cells. Of these, only a few need be mentioned. *Osborne method:*[928] Five hundred grams of pressed yeast are rubbed with 500 cc. of 95 per cent alcohol and allowed to stand at room temperature for sixteen to twenty-four hours. The alcohol is then removed by suction, 500 cc. of water containing 1 per cent chloroform are added and the mass allowed to stand at 30° to 35°C. for six days, with occasional shaking. The extract is then filtered through several folded pieces of filter paper and the filtrate added to 600–700 cc. of 95 per cent alcohol. The precipitate is filtered off, washed with absolute alcohol and dried in vacuo over sulfuric acid. To purify the enzyme still further, 20 grams of this dried material are rubbed up in a mortar with warm water to a uniform suspension, then transferred, with 500 cc. of warm water, to a stoppered cylinder and allowed to stand six to eight hours with frequent shaking. The slimy residue is then filtered off and washed a few times with warm water. The filtrate and washings are then treated with ammonia to remove the magnesium and phosphoric acid. The precipitate is filtered off and the filtrate, to which a small amount of chloroform has been added, is dialyzed against distilled water for eight days. The contents of the dialyzer are then evaporated in vacuo, at 25° to 30°C., to a small volume and treated with absolute alcohol and ether. A flaky, brownish precipitate is obtained, which is filtered, washed with absolute alcohol and dried in vacuo over sulfuric acid.

A very active invertase solution can also be prepared by allowing 25 grams of yeast to decompose in 150 cc. of water. The invertase activity of the liquid continues to increase and reaches a maximum. When the reaction has become acid to litmus and neutral to methyl orange the solution is filtered through infusorial earth until clear.[266] This solution, treated with toluol, will keep its activity for a long time. This extract can invert twenty times its volume of 5 per cent cane sugar solution, at 50°C., in about one hour.

An active preparation of invertase can be made by grinding 100 grams of yeast with sand.[821] The grinding should not be as thorough as is required in preparing zymase. The mass is then shaken with 200 cc. of water containing 1 per cent of chloroform for three–six hours, and then filtered or centrifuged. The extract need not be completely clear. Fifteen to 20 grams of kaolin, to adsorb the proteins, for every 100 cc. of extract are then added with continuous stirring; the reaction should be kept acid. The enzyme solution is then filtered, pouring back the first portion of the filtrate. The solution may then be dialyzed in the presence of chloroform. The dialysate is next poured into three volumes of 95 per cent alcohol, the precipitate filtered, washed with alcohol and dried in vacuo over sulfuric acid.

Another method for the preparation of invertase consists in allowing 2 kgm. of brewer's yeast to autolyze for 12 days and then adding a concentrated solution of lead acetate (containing 70 grams) to every 500 cc. of the autolysate.[392] The mixture is then treated with kaolin and the liquid filtered off by suction. The filtrate is next saturated with hydrogen sulfide to remove the lead; the lead sulfate is removed by the further addition of kaolin. The filtrate is treated once with a small amount of animal charcoal and three times with increasing quantities of kaolin and filtered after each treatment. Finally the filtrate is treated with alcohol. Usually 7.7 grams of a pure white powder are obtained by this method.

Invertase can be prepared from pressed yeast which has been allowed to autolyze for fourteen days at 18°C. in the presence of toluol.[398] The resulting liquid is filtered several times through a folded filter paper. About 300 cc. of juice, which contain one-fourth to one-fifth of the total invertase of the fresh yeast, are obtained from 1 kgm. of fresh yeast. Three cubic centimeters of 4 N acetic acid and 1.6 liters of 95 per cent alcohol are added to every 100 cc. of juice and the mixture actively shaken. The resulting precipitate is filtered, washed with absolute alcohol and ether and dried in vacuo.

A very pure invertase solution may be obtained from yeast by rapid autolysis in the presence of a large amount of water, acetic acid ester and an antiseptic (toluol or chloroform).[1279] The autolysate is neutralized with ammonia or ammonium phosphate, a small amount of kaolin added to adsorb the proteins and the enzyme solution filtered. The invertase is then adsorbed from the solution by adding an excess of aluminium hydroxide. (To prepare this hydroxide, a boiling solution of pure aluminium sulfate or ammonium alum is added to a 15 per cent ammonia solution, heated in an enameled pot, and kept hot for twenty-four hours; $Al(OH)_3$ of a high adsorptive capacity is formed. It is then washed by decantation for a week until free from ammonia, as shown by Nessler's reagent). Quantitative extraction of the invertase from the $Al(OH)_3$ adsorbate can be obtained with 1 per cent aqueous Na_2HPO_4, 1 per cent $(NH_4)_2HPO_4$, 0.04 per cent NH_4OH, 1 per cent $(NH_4)_2C_2O_4$, very dilute Na_2CO_3 or aqueous C_5H_5N. The invertase can also be redissolved in 10 per cent ammonia, filtered, centrifuged and again filtered through the same filter. The filtrate is evaporated in vacuo at 25°C. and dried in a Faust-Heim apparatus. The enzyme is then precipitated with ice cold acetone at 0°C., centrifuged and washed with acetone (this last process should not occupy more than five minutes). The enzyme is redissolved in 0.04 N acetic acid, adsorbed by kaolin and redissolved in 0.2 N Na_2CO_3. The inorganic salts can then be removed by dialysis through a fish bladder. The enzyme solution is then evaporated in vacuo.

Purified yeast invertase of a high degree of activity has approximately the following percentage composition: hydrogen, 6.59–6.88; nitrogen, 11.3–12.5; carbon, 49.70–50.32 (all on ash free basis); cystin (calculated from sulfur content), 2.0; histidine, 2–6 and tryptophane, 5.5.[406,412,872] The latter value is higher than that of native proteins or yeast protein. However, the nitrogen content depends upon the purity of the preparation. Pure preparations contain very little ash and are practically free from phosphorus. The purified enzyme still has colloidal properties and its isoelectric point is at pH 5.0. The accompanying substances (yeast gum, etc.) protect the enzyme from destruction and influence the physicochemical activities of the enzyme. The invertase molecule probably consists of a chemically functioning active group and a colloidal carrier.[1289]

Methods of measurement. Invertase was first described in 1860 by Berthelot[127] as "ferment inversiv" or inverting ferment, because dextro-rotatory cane sugar was transformed, under the influence of the enzyme, into laevo-rotatory invert sugar. The action of invertase can be measured, qualitatively as well as quantitatively, by changes in the polarization of light and in the reduction of Fehling's solution. Cane sugar rotates polarized light to the right, while invert sugar, which consists of a mixture of glucose and fructose, rotates it to the left. Pure cane sugar does not reduce Fehling's solution, while invert sugar does. When invertase, therefore, acts upon a solution of cane sugar, the glucose and fructose produced leads to a diminution of the polarization of light to the right, followed by a polarization to the left and also causes a reduction of Fehling's solution.

Polariscopic method. The determination of the time required to reduce the polarization of sugar-enzyme mixture to 0° under optimal conditions is an excellent quantitative method of estimating the strength of invertase preparations.[933] This measurement may be made as follows:[412] 0.05 grams of dry yeast or purified enzyme are suspended or dissolved in 5 cc. of 0.5 N NaH_2PO_4. This solution is then added to 20 cc. of a 20 per cent sucrose solution (4 grams sucrose) and incubated at 15.5°C. The time (t) required (the mutarotation should be destroyed by the addition of sodium carbonate to each sample) to change the rotation to 0° is determined; this corresponds to an hydrolysis of 75.75 per cent of the sugar. The more concentrated the enzyme, the smaller is the time value (t) of the preparation. The inversion capacity of the enzyme may be calculated from the inversion constant of a known sucrose solution, the number of grams of sugar converted and the number of grams of enzyme used.[1282]

The activity of invertase can also be measured by the following method:[581] 500 cc. of 10 per cent sucrose and 125 cc. of a liquid pressed from fresh beer yeast (or any other enzyme-containing fluid) are incubated at 30°C. Immediately before incubation and at intervals of thirty minutes during incubation, 50 cc. of the mixture are removed and added to 5 cc. of M/5 Na_2CO_3. The Na_2CO_3 destroys the invertase and stops the mutarotation. After standing seven to 120 minutes the rotation of these samples is determined in a polariscope. With an active invertase preparation, the rotation will change from +24.50° to −1.49° in one hundred and thirty minutes. The method can be somewhat improved by making up the 10 per cent sucrose in a M/10 phosphate mixture, which has the optimal reaction for the particular enzyme used, and by adding the 50 cc. sample to 0.5 to 1.0 gm. of basic lead acetate in addition to 5 cc. of M/5 Na_2CO_3. The proteins which are precipitated by the lead acetate should be filtered off before the polariscope reading is made.

The rate of inversion is of the unimolecular order:

$$K = \frac{1}{t} \log \frac{r_0 - r_\infty}{r - r_\infty}$$

in which r_0 is the rotation at the commencement of incubation, r_∞, the rotation at complete inversion, r, the rotation at time, t, and K, the velocity coefficient. This equation may also be expressed as follows:

$$K = \frac{1}{t} \log \frac{100}{100 - x}$$

in which x is the percentage of inversion at time, t.

Picric acid method.[1254] One cubic centimeter of the extract to be tested is added to 8 cc. of water in a 50 cc. centrifuge tube and warmed to 40°C. One cubic centimeter of a 1 per cent solution of cane sugar is then added to the centrifuge tube and incubation at 40°C. continued for thirty minutes. The tube is then rapidly cooled and 0.5 to 1 gram of dry picric acid, or preferably sodium picrate, added. After thorough mixing, the solution is centrifuged and filtered. As soon as possible, the amount of inverted sugar in 3 cc. of the filtrate is determined colorimetrically.[862]

Optimal conditions for the action of invertase. The optimal zone for the action of yeast invertase is between pH 3.5 and 5.5 with the maximal activity at pH 4.5.[826,1158] Honey invertase has its optimum at pH 5.5 to 6.3, the higher value being favorable at the initial stages of the reaction.[876] The optimum for intestinal invertase lies between pH 5.0 and 7.0, as shown by Figure 5. The optimal reaction for bacterial invertase (pneumococci) is pH 7.0.[55] The optimal temperature for invertase is 52°C.

Action of chemicals. Oxidizing agents, such as ozone and iodine, have an injurious effect upon invertase.[406] The salts of heavy metals (mercury, silver and copper) have a very inhibitory action, which is followed by self regeneration of the enzyme after these chemicals are removed.[411] The cell constituents exert a protective action against the inactivating agent, so that purified preparations are more readily inactivated. Various amines, especially p-toluidin, exert an inactivating effect upon invertase. The concentration of sugar and the reaction are of importance; a lower concentration of sugar and a lower acidity favoring inactivation. The inactivation by formaldehyde is weaker than that by amines. Invertase is not sensitive to alcohol at room temperature, but is rapidly destroyed by it at 45°C. Alcohol and acetone have an irreversibly destructive effect, the maximal action of alcohol being with 50 per cent alcohol by volume. In 20 and 80 per cent concentrations, alcohol has no destructive action.[582] Cane sugar exerts a protective action against alcohol, 6 per cent of the sugar reducing the rate of destruction, in 50 per cent alcohol, to one per cent of its original value. Invertase is not very sensitive to the action of neutral chlorides, the sulfates of alkalies, alkali earths and rare earths. The salt effect of sodium chloride seems to approach zero at the optimal reaction, the effect increasing on either side of the optimum.[418] The rate of destruction of invertase by acids and alkalies, at 30°C., follows the law of a monomolecular reaction.[582] Information on the kinetics of invertase reactions may be obtained elsewhere.[245,266,626,846]

b. Maltase. Maltase hydrolyzes maltose to two molecules of glucose according to the formula:

$$C_{12}H_{22}O_{11} + H_2O = 2C_6H_{12}O^6$$

Occurrence. The majority of animal tissues and organs which are capable of hydrolyzing starch also contain maltase so that a part of the maltose, formed from the decomposition of starch by diastase, is converted into glucose. Maltase is found in intestinal secretions, saliva, pancreatic juice, blood and liver,[696a] in extracts of both green and kilned malt,[731] as well as in ungerminated barley. In the last, this enzyme cannot be extracted with water. In plants, especially in malt, the amount of maltase is relatively smaller than that of diastase, so that, when malt diastase acts upon starch, the sugar accumulates chiefly in the form of maltose. Maltase is also formed by various fungi and bacteria. Most yeasts contain maltase, but in different amounts. A few yeasts, such as *Saccharomyces marxianus*, *S. exiguus*, *S. apiculatus*, and *Willia anomala*, do not produce maltase.

Preparation. Eighty-eight grams of washed and pressed bottom yeast are shaken with 132 cc. of water containing chloroform (equivalent to 20 grams of dry yeast and 200 cc. of water). The liquid is neutral at first, but after a few minutes becomes acid. It is advisable to keep the solution at the neutral point, by adding carefully a dilute solution of ammonia. Usually 32.5 to 41.8 cc. of a 1 per cent solution of ammonia are required during a digestion of six hours. It is often more convenient to keep the liquid at the neutral point by adding magnesium cabonate. After six to twenty-four hours' digestion, the maltase solution is filtered.[1276] The enzyme can be precipitated from the filtrate by the addition of alcohol, and the precipitate can be dialyzed to remove the sugars and ash. Nearly 95 per cent of this material can thus be removed in about seventy-two hours. An active maltase can be prepared from corn by extracting the crushed, oil-free, grain with 0.04 per cent tartaric acid in dilute alcohol and then precipitating with concentrated alcohol.[116] In working with maltase solutions, it is important to keep them at 0°C., not merely in an ice box, since maltase is less stable than invertase. At 30°C., maltase disappears in twenty-four hours, but at 0°C., it can be kept for a week. By the use of AlO_2H (prepared by the action of NH_4OH on $Al(OH)_3$ at 250°C.), maltase can be readily adsorbed; this should be followed by elution with $(NH_4)_2HPO_4$. KH_2PO_4 will dissolve the invertase completely and exclusively, while the maltase is left and can be recovered from the adsorbate.[1289c]

Measurement. The presence of maltase can be demonstrated *qualitatively* by polariscopic determinations, phenylhydrazine tests and reduction tests with Barfoed's reagent.[1298] The specific rotation of maltose (α_D) is $+ 137.0°$, and of glucose (α_D) is $+ 52.5°$. In the presence of maltase, the polarization of a maltose solution progressively diminishes.

The phenylhydrazine test[881] is based upon the fact that maltosazone rotates polarized light to the right and glucosazone rotates it to the left, i.e., the rotation (with sodium light) of 0.2 gram of maltosazone in the pyridine-alcohol mixture is +1° 30′ while the rotation of 0.2 gram of glucosazone is −1° 30′. After maltase has acted upon a maltose solution, the protein in solution is removed by heating with a few drops of acetic acid and a few grains of sodium chloride and then filtering. The protein-free filtrate is treated with 2 parts of a colorless phenylhydrazine hydrochloride solution and 3 parts of aqueous sodium acetate. There should be 3 molecules of phenylhydrazine for 1 molecule of maltose. The solution is then warmed over a water bath for one to one and one-half hours. If the solution contains glucose, the glucosazone will separate out during the process of heating, since it is only slightly soluble in water, while the more soluble maltosazone appears only after the liquid has cooled. The crystals of the glucosazone may be separated by quickly filtering the hot liquid. Recrystallization and purification of the crystals can be accomplished by adding pyridine in ligroin ether or benzol and then drying the solution in a desiccator. The rotation of 0.2 gm. of the purified crystals dissolved in 2 cc. of pure pyridine and 6 cc. of absolute alcohol is then determined in a 10 cc. polarimeter tube with sodium light. If the rotation is between 1° 30′ and −1° 30′, the solution is a mixture of maltose and glucose.

Glucose reduces Barfoed's reagent (1 per cent solution of copper acetate containing a small amount of acetic acid), but maltose does not. When the latter has been hydrolyzed, it is able to reduce Barfoed's reagent.[1298]

Maltase can be measured *quantitatively* by determining the degree of polarization or the amount of reduction of Fehling's solution. Five cubic centimeter portions of the enzyme preparation should be added to 5 cc. portions of a 10 per cent maltose solution and 0.5 cc. of toluol in small flasks and incubated at 30°C.[686a] Immediately after mixing and after various periods of incubation, 2 cc. portions of the mixture are taken out, freed from protein by pouring into 25 cc. of a 5 per cent zinc acetate solution in 95 per cent alcohol. The solution is then made up to 50 cc. with alcohol, shaken, filtered and the rotation determined. The enzyme solution, without the maltose, is treated in a similar way as a control. The degree of rotation of this control should be subtracted from that of the maltose-enzyme mixture.

Since 1 cc. of Fehling's solution is reduced by 0.00778 grams of maltose and by 0.005 grams of glucose, the hydrolysis of maltose by maltase is accompanied by an increase in reduction. Ten or 20 cc. of the enzyme solution are added to 50 cc. of a 5 per cent maltose solution containing a small amount of toluol and then incubated. The amount of reducing sugar in 10 cc. portions of the mixture, after freeing it from protein, is determined immediately before incubation and at intervals during incubation.

Optimal conditions for activity. Yeast maltase has its optimum at pH 6.1 to 6.8 (6.6),[833] while *Asp. oryzae* maltase acts at a much greater acidity, even at pH 4.0.[406] The optimal temperature is 40°C., and the enzyme is destroyed at 55°C.[733] The optimal temperature for the maltase of *Asp. oryzae* (47°C.) is independent of the concentration of enzyme and substrate; at an acid reaction, the enzyme is rapidly destroyed at this temperature.[270,273] Maltase is very thermolabile and is rapidly destroyed at reactions within narrow limits of its optimum, even at room temperature. Neutral salts have practically no effect upon the activity of maltase. Yeast maltase is very sensitive to alcohol, being injured by even 5 per cent; if alcohol is employed in precipitating the enzyme from solution, it should be removed as soon as possible. Maltase becomes inactive when the yeast is dried in the air and will retain its activity for only a few days in aqueous solution. Maltase prepared from sources other than yeast, as for example from *Asp. oryzae,* is less sensitive.[159] Information on the relative specificity of yeast maltase,[1285] and on the influence of antiseptics[682] may be obtained elsewhere.

c. Trehalase. Trehalase hydrolyzes the disaccharide trehalose, which is present in Syrian manna and in the mycelium of fungi, into two molecules of glucose. It is sensitive to acids and alkalies. Its upper temperature limit is 64°C. The optimal reaction is at a slight acidity.[176] Trehalase has been demonstrated in the intestinal secretions of certain animals (horse, cattle), certain fishes, in malt, in various fungi, in yeasts and in certain bacteria. The enzyme is obtained by precipitating a water extract of the crude material with alcohol, or by using an acetone-ether preparation of certain fungi (*Asp. niger*). The action of the enzyme can be determined by means of the polariscope or reduction tests with Fehling's solution.

d. Lactase. Lactase which converts lactose to glucose and galactose was first discovered in milk sugar yeasts;[115] it was demonstrated that light-forming bacteria were active upon lactose media only when a small amount of these yeasts had previously been added so as to produce glucose for the activities of the bacteria. Maltase and lactase are present in various species of yeast but have not, as yet, been observed together in the same yeast. Some fungi, however, may produce both enzymes. Lactase is formed by several fungi, such as certain Mucorales, Aspergilli and Penicillia, various plants, and bacteria. Kefir lactase, which is injured by the addition of galactose (galacto-lactase), has been differentiated from almond lactase, which is injured by the addition of glucose (gluco-lactase).[44] Lactase is also present in the intestinal secretions, membranes and faeces of infants and young animals. The enzyme is found in the intestinal canal of the foetus, and its amount diminishes progressively during foetal life.[980] The lactase content may entirely disappear in older animals, although it may reappear if lactose is fed.

Preparation. Lactase may be prepared by extracting with water, saturated with ether, the cleaned membrane of the small intestines of suckling animals or those previously fed on lactose.[980] Animal lactase can also be obtained by grinding these cleaned intestinal membranes with sand and extracting with five volumes of physiological salt solution. The extract is then filtered and precipitated with 5 volumes of 95 per cent alcohol. This precipitate is washed with ether and dried over $CaCl_2$. Plant lactase is obtained by grinding kefir grains with water for one-half to one hour and then allowing the suspension to stand at room temperature. The lactase, as well as invertase, goes into solution.

Since lactase cannot be measured directly in fresh yeast, it must be separated from the cells. Yeast lactase can be prepared by macerating 5 grams of fresh yeast (*S. fragilis*) for 10 minutes with 1 cc. of chloroform, then diluting with 7 cc. water and carefully neutralizing with 1.1 cc. of 1 per cent ammonia.[1280] After 6 hours, the liquid becomes markedly acid, requiring 0.3 cc. of ammonia for neutralization. After three days the lactase solution is filtered and 77 per cent of the enzyme is obtained in solution.

Methods of measurement. Lactase may be demonstrated *qualitatively* by the rotation of polarized light, the phenylhydrazine reaction, the reduction of Barfoed's reagent and the fermentation test. The specific rotatory power of lactose ($\alpha_D = +52.5°$) is the same as that of glucose ($\alpha_D = +52.5°$) and is considerably less than that of galactose ($\alpha_D = +81.5°$). Since one molecule of lactose decomposes into one molecule of glucose and one molecule of galactose, the presence of lactase will be indicated by an increase in the dextro-rotatory power. The phenylhydrazine reaction is based upon the fact that phenyl-lactosazone (0.2 gram dissolved in pyridine-alcohol mixture) is practically inactive optically while the rotation of glucosazone is −1°30′ and that of galactosazone is −0°48′. The determination is performed similarly to that for maltase. The reduction test with Barfoed's reagent is also similar to that for maltase, since lactose does not reduce Barfoed's reagent while glucose does. The fermentation test is based upon the fact that pressed yeast does not ferment pure lactose, unless the latter has first been converted into glucose as a result of the action of lactase. The formation of carbon dioxide by the yeast indicates the previous action of lactase.

For *quantitative* purposes, the following method is very useful:[979] A freshly prepared lactase solution is added to 5 grams of lactose dissolved in 80 cc. of water, thoroughly shaken, made up to 100 cc. with water, and, after adding 1 cc. of toluol, incubated for twenty-four hours. Another flask containing the same ingredients except that the enzyme solution has previously been boiled is used as control. After twenty-four hours, each solution is cooled, transferred quantitatively to a measured 1 liter flask, clarified by the addition of a few drops of mercuric nitrate, neutralized with a dilute solution of sodium carbonate and the volume made 1 liter with distilled water. The solutions are then filtered, the excess of mercury precipitated with pulverized zinc and the solutions again filtered. The amount of solution, in both enzyme and control flasks, required to reduce 10 cc. of Fehling's solution is determined. A curve has been constructed from which the amount of lactose hydrolyzed can be readily determined.[979]

The action of lactase may also be measured as follows:[1280] 10 cc. of M/3 phosphate mixture (3.8 cc. primary and 6.2 cc. of the secondary salt, prepared according to Sörensen) are added to 25 cc. of a 10 per cent lactose solution. After introducing the enzyme, sufficient water is added to make the volume 50 cc. The reacting mixture is then incubated at 30°C. Five cubic centimeter portions of the mixture are removed at various intervals and added to 5 cc. of 2 N sodium carbonate solution. One-fifth of this mixture is used for copper determination by Bertrand's method.[130b] The number of minutes required for 1 gram of dry yeast, or an equivalent enzyme solution, to hydrolyze completely 2.5 grams of lactose at 30°C. and pH 7.0, is taken as a unit of the activity of lactase.

Optimal conditions. The optimal reaction for almond lactase is pH 4.2 to 4.6; at pH 3.0, the enzyme is still very active. Yeast lactase, however, has its optimum at about pH 7.0. Lactase is most active at 36°C. As it is destroyed by exposure to 45°C. for three hours,[44] the preparation of the enzyme should be carried out at low temperatures.

e. Melibiase. This enzyme hydrolyzes melibiose, which is obtained from raffinose, to glucose and galactose. It is produced by certain bottom yeasts. Its action is most easily determined by the polariscope, since melibiose is markedly dextro-rotatory ($\alpha_D = +129.38°$) and its decomposition leads to a decrease in polarization. The optimal temperature is 50°C. and the destructive temperature 70°C.

f. Cellobiase (*cellase*). Cellobiase converts the disaccharide, cellobiose, which is obtained from the decomposition of cellulose, into two molecules of glucose. (Cellulose is probably a polymer of anhydro-cellobiose.[991]) Cellobiase occurs in the emulsin of almonds and other seed, in various grains and fruits, kefir corn and fungi.[129b, 443] Malt contains both lichenase and cellobiase, the former hydrolyzing lichenin to cellobiose.[994] The enzyme is often prepared by growing *Asp. niger* in a nutrient solution containing asparagine, drying carefully, and extracting the mass with 10 times its weight of water for twenty-four hours at 37°C. For the demonstration of the enzyme, 10 cc. of this solution are added to 0.5 gram of cellobiose. After various periods of incubation, the amounts of glucose produced are determined by tests with Fehling's solution. The optimal reaction is pH 6.0[130] and the optimal temperature 46°C.

g. Gentiobiase. Gentiobiase hydrolyzes the disaccharide, gentiobiose, which is formed during the decomposition of the trisaccharide, gentianose, into two molecules of glucose.[1321a] Gentianose can also be converted by gentiobiase into cane sugar and glucose. Gentiobiase is produced by bottom yeasts, certain fungi[175] and invertebrate animals.[139] It is always found accompanying lichenase and cellobiase and is well distributed in the plant kingdom.[642] The method for the determination of gentiobiase is similar to that described for cellobiase.

4. ENZYMES ACTING UPON GLUCOSIDES AND GLUCOSE COMPOUNDS.

a. Emulsin (*β-phenol-glucosidase or prunase*). Enzymes of the emulsin type are capable of hydrolyzing β-glucosides and various sugars, such as raffinose and stachiose. The term emulsin is usually applied to a definite enzyme preparation such as yeast extract and designates a mixture of enzymes rather than a single enzyme.

Occurrence. Emulsin is widely distributed in the plant kingdom and is especially abundant in sweet and bitter almonds, the seed of various *Prunaceae*, such as *Prunus domestica*, and the *Rosaceae*. Emulsin is found not only in the seed but also in the roots, stems and leaves of various plants.[555] It has also been demonstrated among the cryptogamic plants, in fungi[168,177] and in yeasts.[553,885] It does not occur in higher animals, but is widely distributed among the invertebrates.

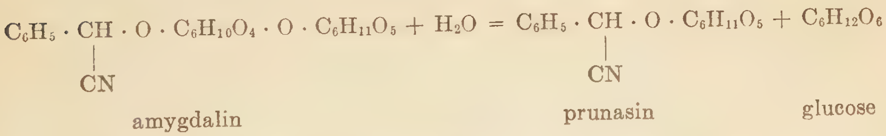
Preparation. One hundred grams of sweet almonds, dipped for a minute in boiling water, are freed from the skins, finely ground and then mixed with 200 cc. of distilled water saturated with chloroform.[555] After standing twenty-four hours, the liquid is pressed out, yielding about 150 cc. of extract. The protein impurities are removed by precipitation with a few drops of acetic acid, followed by filtration through paper. The emulsin in the filtrate is then precipitated with 500 cc. of 95 per cent alcohol. The precipitate is washed with a mixture of alcohol and ether and dried in vacuo over sulfuric acid; a white powder soluble in water is obtained.

In another method of obtaining emulsin, the almonds should be warmed for fifteen minutes in water, at 60° to 70°C.; the surface of the almonds is then dried by an air current, the almonds ground and the larger part of the oil pressed out by means of an hydraulic press.[1278] The dry residue is extracted with three volumes of ether, ground and again extracted with two volumes of ether. The ether is removed, the material dried in a warm air current and ground in a sieve. The resulting powder after being dried in vacuo weighs about one-third as much as the original almonds. The powder is used as a source of enzyme and is quite stable, losing only 10 per cent of its strength in six months. A purified product can be prepared from the powder by extraction with 2.5 volumes of N/10 ammonia solution, then diluting the extract one-third with water and shaking for five hours. The protein is precipitated from the liquid extract with dilute acetic acid (300 cc. N/10 acetic acid or 60 cc. N/2 for 500 cc. of extract). The filtrate is then treated with one-half of its volume of 95 per cent alcohol. This treatment results in a precipitate consisting chiefly of gum and protein, and containing some of the enzyme. This fraction is removed and the enzyme precipitated from the remaining liquid with four volumes of alcohol. The white precipitate obtained after filtration is washed with absolute alcohol and ether. 2.5 to 3.5 grams of emulsin can thus be obtained from 100 grams of almond powder, which is equivalent to 60 to 75 per cent of the theoretical yield. To purify the enzyme further, it is redissolved and reprecipitated by adding a trace of calcium chloride. This preparation is soluble in water, but the solution is precipitable with hydrochloric acid.

Emulsin can also be prepared from the kernels of plum stones.[547] The stones are milled and the paste extracted with water (containing toluol) for nine weeks. The enzyme can then be precipitated with 95 per cent alcohol, further purified by dialysis and reprecipitated with alcohol. Ethyl alcohol (2 volumes for forty-eight hours), methyl alcohol (2 volumes for thirty-one hours) and acetone (2 volumes for twenty-eight hours) can also be used as precipitants of emulsin.[190] This enzyme can be extracted from fat-free almond powder with dilute ammonia; the proteins are first precipitated with acetic acid, then the enzyme with alcohol or acetone.[634]

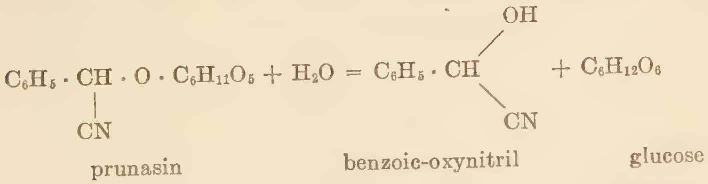
Nature and action of emulsin. Emulsin obtained from almonds probably consists of at least four enzymes:[44,229] (1) *amygdalase* hydrolyzing amygdalin to mandelic acid-nitril glucoside (prunasin) and glucose; (2) *prunase* or *β-phenol-glucosidase* converting mandelic acid-nitril glucoside (prunasin) into benzoic-oxynitril and glucose; (3) *oxynitrilase* and (4) *lactase*. In addition, cellobiase, melibiase and other enzymes are often present in preparations of emulsin.[1278]

Amygdalase acts as follows:



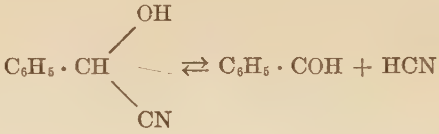
$$\underset{\text{amygdalin}}{C_6H_5\cdot\underset{\displaystyle CN}{\underset{|}{CH}}\cdot O\cdot C_6H_{10}O_4\cdot O\cdot C_6H_{11}O_5}+H_2O=\underset{\text{prunasin}}{C_6H_5\cdot\underset{\displaystyle CN}{\underset{|}{CH}}\cdot O\cdot C_6H_{11}O_5}+\underset{\text{glucose}}{C_6H_{12}O_6}$$

The β-glucoside, prunasin, is then hydrolyzed by *prunase:*



$$\underset{\text{prunasin}}{C_6H_5\cdot\underset{\displaystyle CN}{\underset{|}{CH}}\cdot O\cdot C_6H_{11}O_5}+H_2O=\underset{\text{benzoic-oxynitril}}{C_6H_5\cdot CH\begin{matrix}\diagup OH\\ \diagdown CN\end{matrix}}+\underset{\text{glucose}}{C_6H_{12}O_6}$$

Finally the third enzyme (*oxynitrilase* or *benzcyanase*) converts benzoic-oxynitril into benzaldehyde and hydrocyanic acid and can also synthesize it from these two components:



$$C_6H_5 \cdot CH\begin{matrix} OH \\ CN \end{matrix} \rightleftarrows C_6H_5 \cdot COH + HCN$$

The synthesizing enzyme has been called syn-emulsin to differentiate it from dia-emulsin, which hydrolyzes the β-glucoside.[1052]

Other enzymes acting upon β-glucosides of the nitril-β-glucoside type are *linase*, or *phaseolunatase*, which is found in *Linum usitatissimum* and in *Phaseolus lunatus* and which hydrolyzes linamarin (acetonoxynitril-glucoside),[44, 355] and *vicianinase* which converts vicianin, present in various species of *Vicia*, into a gluco-arabinose, hydrocyanic acid, and benzaldehyde.[129]

Measurement. All the glucosides digested by emulsin are laevorotatory. Since d-glucose is always formed as a result of the hydrolysis produced by the enzyme, a change in rotation from left to right occurs and the degree of this change is an index of the concentration of the enzyme. The amount of the sugar formed can also be determined by reduction tests with Fehling's solution. Similar methods can be applied to the study of the hydrolysis of nitrilglucoside. The digestion of nitrilglucoside can also be measured by titrating, with a solution of iodine and sodium carbonate, the amount of hydrocyanic acid liberated.[54b] It should always be remembered that all three enzymes may be acting at the same time. The quotient obtained by dividing the amount of enzyme used, by the time required for the hydrolysis of 50 per cent of 0.1 gram of amygdalin has been suggested as a unit of enzymatic activity, by which different preparations can be compared.[1278] The activity of emulsin can also be measured as follows:[548] A salicin solution is made by dissolving 2.5 grams of salicin in 60 cc. of water, then adding 12.5 cc. N/10 sodium acetate and 10 cc. N/10 acetic acid, and making up the volume to 1000 cc. at 0°C. with water. Ten milligrams of emulsin are then moistened with 1 cc. of water and, after ten minutes, 4 cc. of the salicin solution are added and the mixture incubated. The amount of glucose formed after various intervals is then determined.

Optimal conditions. The optimal temperature for emulsin is 45° to 50°C. Its optimal reaction is near neutrality. Emulsin is inactivated by acids and is destroyed by alkalies. The optimal reaction for amygdalase is pH 6.0 and for prunase pH 4.4.[1278] The optimum for β-glucosidase varies from pH 4.1 to 4.4 depending upon the nature of the substrate.[634]

An attempt has been made to divide the emulsins on the basis of the particular glucosides, upon which they act, i.e., *arbutase, äskulase, primiverase, elaterase, populase, phlorizinase, salicase, rhamnase* and *indemulsin*, the enzyme which forms blue indigo from the natural glucoside of indigo plants.[923]

b. Myrosinase (*sinigrinase*). This enzyme which is present in black mustard seed and various other *Cruciferae* occupies a place apart from emulsin. It hydrolyzes the glucoside, myrosin (sinigrin or potassium myronate), to oil of mustard, glucose and potassium bisulphate:

$$C_{10}H_{16}O_9NS_2K + H_2O = C_3H_5{\cdot}N{:}CS + C_6H_{12}O_6 + KHSO_4$$

Myrosinase may be obtained from several organs of various *Cruciferae*, especially from the seed. It is also found in the seed and plants of *Resedaceae*, *Violaceae* and *Tropaeolaceae* but not in microörganisms.[1160] The enzyme is prepared by extracting 200 grams of ground white mustard seed with water, then precipitating the extract with an equal volume of alcohol.[1097] The precipitate is extracted three times with ether to remove the mustard oil. The acid enzyme solution is then neutralized. The mustard oil formed, as a result of the hydrolysis of the sinigrin, has a characteristic odor. This oil has been used for the demonstration of the presence of the enzyme.[1160] If the odor of mustard oil is present in the original plant extract, the oil is first removed by warming or extracting with ether. The activity of the enzyme may also be determined by reduction tests with Fehling's solution, since glucose is one of the end-products of the reaction. The determination of the amount of $KHSO_4$ formed can also be utilized as an index of enzyme action;[1188] the solution is titrated before and after incubation with a N/50 alcoholic solution of sodium hydroxide using carminic acid as an indicator. The optimal temperature is 45° to 50°C.

c. Nucleosidase. Nucleosidase converts nucleosides to ribose and adenine.[722–724] The enzyme occurs in the kidney, spleen and pancreas of cattle. A liquid extract is prepared by treating the minced organs with a 10 per cent sodium chloride solution (or phosphate buffer solution), then pressing out the juice with an hydraulic press. The juice can then be concentrated in vacuo; the syrupy liquid may either be evaporated to dryness or precipitated with acetone. The enzyme can be purified by precipitation with dialyzed iron and alcohol. The action of the enzyme is measured by the amount of ribose liberated. The adenine must first be removed with phosphotungstic acid or mercuric acetate. The optimal reaction is pH 7.5 and the optimal temperature 37°C.

d. Tannase. Tannase is the enzyme which converts tannin (galloyl-glucose) to gallic acid and glucose:

$$3C_{18}H_{16}O_{12} + 6H_2O = 6C_7H_6O_5 + 2C_6H_{12}O_6$$

Although glucose is liberated, it is not from a glucoside, and the enzyme is, therefore, distinct from emulsin.[442] The action of tannase is probably related to that of an esterase.[461] Various fungi of the *Aspergillus* and *Penicillium* groups, when grown on media containing tannin, form the enzyme, tannase;[437,983] the higher the concentration of tannic acid in the synthetic medium, the greater is the production of tannase by the culture of fungi.[669] The maximal amount of enzyme is formed when the sugar of the medium is completely replaced by tannic acid. Gallic acid may take the place of tannic acid.[1014]

Preparation. Tannase can be prepared by boiling 600 grams of coarsely ground myrobalans for ten minutes in 3000 cc. of tap water.[461] The liquid is then poured off and the residue extracted with 2000 cc. of hot water. This extraction is repeated three or four times. The combined extracts are then added to 300 grams $(NH_4)_2SO_4$, 9 grams K_2HPO_4 and 3 grams $MgSO_4$ dissolved in water and the total volume made up to twelve liters with water. This liquid is then poured in dishes to a depth of 4 cm., inoculated with *Asp. niger* and incubated in the dark, at 28° to 43°C., for four days. After this incubation the fungus mass is lifted out, thoroughly washed six times with water, and pressed out by hand. The moist fungus material is ground up with 1000 cc. of water and 1 cc. of toluol, and allowed to stand for twenty-four hours at 20°C. Acid is formed in eight to ten hours and should be carefully neutralized every two to three hours thereafter with a cold saturated solution of barium hydroxide. The extract is then filtered through a layer of infusioral earth, and the mycelium again extracted with 500 cc. of water containing 0.5 cc. of toluol for two hours. The total extract is concentrated in vacuo to 50–60 cc., filtered through infusorial earth and treated with five volumes of absolute alcohol to precipitate the tannase.

The enzyme is redissolved in 20 to 30 cc. of water and reprecipitated with five volumes of absolute alcohol, washed with ether and dried in a desiccator. If the resulting light green powder reduces Fehling's solution, the precipitation should be repeated again.

Tannase is active in a slightly acid solution but is injured by an excess of acid. The action of tannase can be measured with a polariscope or by determining the increase of acidity, but both methods are very imperfect since several substances of unknown polarization values are formed and the increase of acidity is negligible in well buffered solutions.

Among the other hexosidases only the following need be mentioned: α-methyl-glucosidase and other α-alkyl-glucosidases; β-methyl-glucosidase and other alkyl-β-glucosidases, phenol-β-glucosidases (arbutase, salicase, äskulase and other natural glucosidases); alkyl-β-galactosidases and the enzymes acting on fructosides, sorbosides, maltosides, pentosides and rhamnosides.[406,923]

CHAPTER XI

ENZYMES ACTING UPON PROTEINS AND THEIR DERIVATIVES

1. COAGULATING ENZYMES. *a. Blood coagulation.* Blood coagulation is sometimes considered to be the result of the action of a specific enzyme upon the fibrinogen in the blood, by which it is converted into the fibrin of the blood clot. Although the enzymatic nature of this process is often questioned and the phenomenon considered to be purely colloid-chemical in nature[847,690a] it will be described here as an enzymatic reaction. The coagulation of blood involves at least two distinct processes (1) the formation of thrombin and (2) the transformation of the soluble fibrinogen of the blood into insoluble fibrin. The theories of Schmidt,[1089,1090] Bordet[163] and others[847] are in agreement with this general view, while Wooldridge[1304] and Nolf[893] suggest that thrombin is the product of coagulation rather than its cause. The diagrammatic representation in Figure 11 presents the essential features of blood coagulation according to these theories. One of the essential features of Howell's[576] theory of blood coagulation is that blood contains an inhibitory substance (*heparin*),[578] which antagonizes the prothrombin; heparin is not an antithrombin, since it does not prevent the reaction between thrombin and fibrinogen, but prevents the activation of prothrombin to thrombin, and thus preserves the fluidity of the blood. Its action is neutralized in shed blood by the phosphatid factor furnished by the blood corpuscles or tissue cells. The exact nature of the processes by which blood coagulation occurs is still a subject of controversy but the usually accepted view is that in the circulating blood there is a substance from which thrombin is derived, designated as prothrombin[576], thrombogen,[467,847,1089,1090] serozyme or proserozyme.[163,557] When blood is shed, the disintegration of the blood corpuscles or contact with the tissues liberates a phosphatid substance, known as thromboplastin, [576], thrombokinase[1089,1090] or cytozyme,[163] which with the calcium and prothrombin of the blood reacts to produce thrombin. The thrombin reacts with the fibrinogen of the plasma to form fibrin. The separation out of fibrin is followed by the gelatinization of the plasma or the formation of the clot.

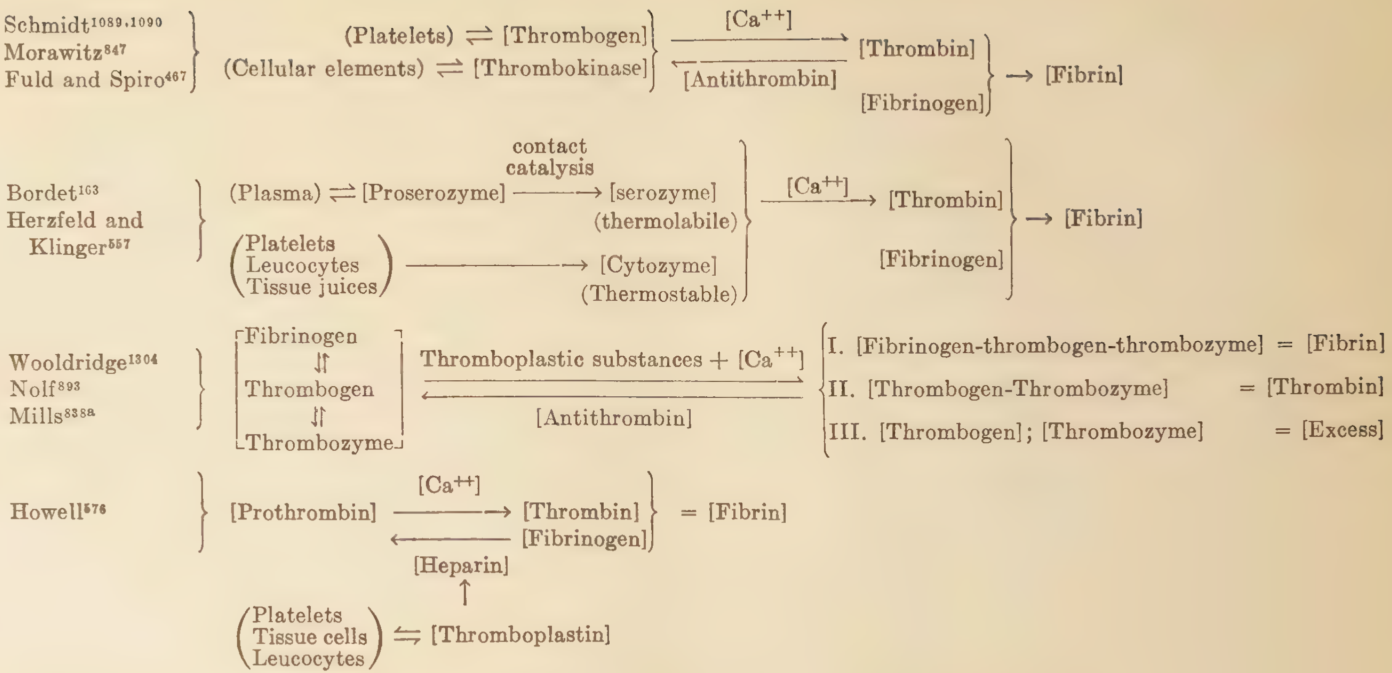

Schmidt[1089,1090]
Morawitz[847]
Fuld and Spiro[467]
(Platelets) ⇌ [Thrombogen]
(Cellular elements) ⇌ [Thrombokinase]
[Ca++]
[Antithrombin]
[Thrombin]
[Fibrinogen]
→ [Fibrin]
Bordet[163]
Herzfeld and Klinger[557]
(Plasma) ⇌ [Proserozyme]
contact catalysis
[serozyme] (thermolabile)
Platelets
Leucocytes
Tissue juices
[Cytozyme] (Thermostable)
[Ca++]
[Thrombin]
[Fibrinogen]
→ [Fibrin]
Wooldridge[1304]
Nolf[893]
Mills[838a]
Fibrinogen
Thrombogen
Thrombozyme
Thromboplastic substances + [Ca++]
[Antithrombin]
I. [Fibrinogen-thrombogen-thrombozyme] = [Fibrin]
II. [Thrombogen-Thrombozyme] = [Thrombin]
III. [Thrombogen]; [Thrombozyme] = [Excess]
Howell[576]
[Prothrombin]
[Ca++]
[Thrombin]
[Fibrinogen]
= [Fibrin]
[Heparin]
Platelets
Tissue cells
Leucocytes
[Thromboplastin]

FIG. 11. MECHANISM OF BLOOD CLOTTING (DIAGRAMMATIC)[690a]

i. *Prothrombin* can be prepared from normal and hemophilic blood by several methods. (1) The acetone method:[576] The blood is oxalated and centrifugalized, and the clear plasma is pipetted off. To each of 5 cc. of plasma are added 5 cc. of acetone. The solutions are well mixed and the resulting precipitate is filtered off quickly by suction and washed once with ether. The filter is removed and opened, and the precipitate spread out in a thin layer by means of a spatula. The filter with its precipitate is dried in a current of air from an electric fan. The dried filters are kept in a desiccator until needed. A prothrombin solution is made by cutting the paper into small pieces and extracting them for twenty to thirty minutes with 10 cc. of water to which two drops of 0.5 per cent sodium bicarbonate have been added. The filtered solution contains the prothrombin. (2) The alcohol method: The oxalated plasma is treated with five times its volume of absolute alcohol and the resulting precipitate filtered off, washed with alcohol and ether, and dried.[807] The final residue is extracted with water and centrifugalized. The supernatant fluid contains the prothrombin. (3) The heat method: The oxalated plasma is heated quickly to 56°C. in a water bath and the resulting precipitate of fibrinogen is centrifugalized off. The supernatant liquid contains the prothrombin. (4) The bacterial method:[493a] The oxalated plasma is inoculated with a fresh culture of Staphylococcus aureus and incubated from six to twelve hours or longer at 37°C. With the growth of the organism the fibrinogen separates out as a clot. This is centrifugalized off; the supernatant liquid contains the prothrombin. If the incubation period is prolonged the clot disintegrates and the fibrinogen settles out as a precipitate.

ii. *Thrombin* is prepared by washing fresh fibrin in a stream of cold water, kneading and picking until all the hemoglobin is removed.[576] The excess of water is squeezed out of the mass, and the latter is cut into small pieces with scissors. This material is covered with sufficient 8 per cent sodium chloride to float it and the preparation is left in an ice chest for forty-eight hours, with occasional stirring. The extract is then filtered through cheese cloth, without any pressure other than the weight of the mass. An equal volume of acetone is added to the filtrate and the resulting precipitate is filtered off as quickly as possible, dried on the filter paper by an air current and kept in a desiccator until needed. The thrombin may be extracted by placing the dried filter paper in water for one hour and then filtering. The filtrate should be clear; it contains the thrombin together with some coagulated protein. For many purposes this preparation is sufficiently pure and may be conveniently kept for future use by being dried in watch glasses. These with their dried contents may be kept indefinitely in a desiccator. When needed, the contents of one or more glasses may be dissolved in water, using such dilution as may be necessary for the purpose in view. If a purer (but weaker) preparation of thrombin is desired the solution obtained above may be dialyzed in a collodium sack, against a large volume of distilled water for thirty to forty-five minutes. The contents of the collodium sack are then filtered and the filtrate shaken with chloroform. The mixture is next filtered (more than once if necessary) until the filtrate is water clear. The filtrate should be free from all traces of coagulable protein. It may be kept by evaporating to dryness in watch glasses, 1 or 2 cc. to each glass.

Thrombin is a protein of the class of pseudo-globulins and is soluble in water. It is thermolabile, being destroyed at 60° to 70°C., and is precipitated by half saturation with ammonium sulfate.[576] Methyl alcohol at 40°C. or higher coagulates it and renders it insoluble. The biuret, Millon's, tryptophane and histidine tests are positive.

iii. *Thromboplastin*,[576] *or thrombokinase*,[1089,1090], *or cytozyme*.[162] Thromboplastic substances are not enzymes but are products of the blood cells, platelets, leucocytes and the tissue cells in general; they have been obtained as lipoid extracts from brain, muscle, liver, lung, spleen, thymus, lymph gland, salivary gland, bone-marrow and Jack bean. A substance having the properties of thromboplastin, namely the lipoid cephalin, has been extracted from dried brain tissue with ether; this extract is then evaporated, precipitated with acetone and washed with alcohol.[576] A similar substance can be obtained from blood platelets, leucocytes, or dry muscle with toluol; the resulting residue is dissolved in alcohol, evaporated and redissolved in toluol.[163] It can also be prepared by covering minced lung with twice its volume of water, adding sufficient acetic acid to make a 15 per cent solution, and centrifuging; the precipitate obtained is then neutralized with 10 per cent sodium bicarbonate.[91] Twenty per cent alcohol is added and the mixture evaporated and dried at 34°C. over sulfuric acid. Thromboplastin may also be extracted from tissue cells and blood corpuscles by water or dilute saline. In these extracts, it is present as a phosphatid-protein complex which is coagulable by heat, but the phosphatid which is the essential constituent, when extracted by lipoid solvents, is thermostable at 100°C. Exposed to the atmosphere, the unsaturated lipoid groups become saturated and there is a consequent loss of the thromboplastic properties. The action of thromboplastin is chemical in nature and not catalytic.

iv. *Antithrombin.* Antithrombin is an albumin-like substance, probably a nucleic acid compound,[347] and is prepared from liver tissue. It is soluble in water, and precipitated at 100°C. in faintly acid solutions but not in neutral solutions. Hirudin which is the typical antithrombin is an albumose compound. Antithrombin, as it occurs in the circulating blood, is distinctly thermolabile, being destroyed at 65 to 70°C. It prevents the action of thrombin upon fibrinogen and, therefore, is distinct from heparin.

v. *Methods of study.* A detailed review of the methods used in the study of blood coagulation may be obtained elsewhere.[576, 1298] All of these are based upon the rapidity of blood coagulation in a capillary tube, in a flask or in drops upon a clean surface. To measure the concentration of thrombin in a particular preparation, increasing quantities of the preparation are added to definite amounts of fibrinogen, and the minimal amount which will produce clotting in a given period of time is determined. The amount of thrombin may be titrated by using fresh serum as a source of thrombin and ammonium sulfate plasma[800a] or magnesium sulfate plasma[1296] as a source of fibrinogen. For the study of the thromboplastic action of various substances (as cephalin), 10 drops of blood plasma (containing 0.2 to 0.25 per cent sodium citrate) are placed in a series of small flat bottomed tubes.[1236] Different amounts of the thromboplastic agent (0.25 per cent solution) and reciprocal amounts of distilled water are then added, followed by the addition of three drops of calcium hydroxide solution to neutralize the excess of citrate in the plasma. The time required for the complete irreversibility of the clot is then determined.

Exposure of the body to Röntgen rays greatly shortens the clotting time of the blood, but no effect is produced by treating the blood in vitro.[732,927] The addition of the serum of menstruating women to human and dog blood delays the clotting time more than normal serum.[768] An extract of the posterior lobe of the pituitary body does not produce coagulation of the blood in vitro but seems to accelerate it.[701] An extract of the anterior lobe markedly delays coagulation unless the extract has been prepared with ether. The injection into an animal of very large amounts of leucoprotease causes an initial delay of blood coagulability and later an acceleration; the addition of this enzyme to drawn blood retards its coagulation.[618] The ingestion of 40 to 60 cc. of a 1 or 2 per cent solution of pectin produces a maximal hemostatic action in 1 to 2 hours, which persists 8 to 12 hours and then gradually declines.[1229] Hemorrhages in many patients have been arrested by this treatment. The same effect has been produced in animals by subcutaneous and intravenous injections of pectin. In children suffering from hemophilia, whose blood has a delayed coagulation time, the intravenous injection of 20 cc. of 5 per cent calcium chloride reduces the coagulation time and decreases the tendency to hemorrhages, but the ingestion of calcium salts has no effect.[235,745] The intramuscular injection of blood, or, even better, a blood transfusion is probably more efficacious in the treatment of hemophilia, but the results of any form of therapy are usually only temporary.

b. Rennet (*lab, chymosin*), *prorennet, antirennet, pararennet* (*parachymosin*). Rennet (lab) will coagulate milk under certain conditions by transforming casein into paracasein in the presence of calcium.[806] The question of the identity of rennet has aroused considerable discussion: according to one view, rennet is identical with pepsin, and the ability to coagulate milk is merely one of the properties of pepsin,[954,962] while others have maintained that rennet is a specific coagulating enzyme, distinct from pepsin.[512] The latter acts best at a strongly acid reaction and rennet at a nearly neutral one. By the action of alkalies, by dialysis and by the use of an electric current, rennet can be freed from pepsin.[220] However, those investigators who oppose the idea of separate enzymes suggest that the above treatment merely results in masking the action either of rennet or pepsin. Hammarsten[512] finally succeeded in obtaining pure rennet by adsorption with casein, the pepsin being left in solution and the rennet coming down in the precipitate. He considers this enzyme as a normal digestive enzyme of the stomach, which acts upon various proteins, when the gastric juice is not sufficiently acid for the action of pepsin. A detailed review of the colloid chemistry of rennet coagulation is obtainable elsewhere.[947a]

Occurrence. Both rennet and pepsin are found in the gastric juice of man, various vertebrate and invertebrate animals and certain fishes, especially in that of calves and other suckling animals. Rennet also occurs in the pancreas and several other animal organs;

the milk-coagulating property of these organs has been ascribed, however, to the action of other proteolytic enzymes.[923] Rennet is formed abundantly by certain higher plants and various micro-örganisms.

Preparation. The enzyme is prepared by extracting gastric mucosa with 0.1 to 0.2 per cent hydrochloric acid for twenty-four hours, at room temperature; the zymogen (*prorennet*) is thus changed into the active enzyme. Glycerin and salt solution can also be used for the extraction of the enzyme. The extract is filtered and carefully neutralized. A small amount of magnesia (1 gm. to 100 cc. of extract) is added and the mixture shaken for a few minutes and then filtered. This process, which should be repeated thrice, greatly reduces the peptic action of the preparation. The filtrate is then slightly acidified and can be concentrated by precipitation with alcohol. The rennet can be still further separated from pepsin by precipitation with an alcoholic solution of cholesterol and a small amount of ether. By adsorbing the enzyme with aluminium hydroxide and redissolving with a phosphate buffer at pH 6.98, the concentration of the rennet can be increased thirty-five times and the nitrogen content of the preparation reduced ten fold.[758a]

Action of rennet. The enzyme can be demonstrated *qualitatively* by adding 1 to 5 cc. of a neutralized extract to 10 cc. of milk and then determining the rapidity of the coagulation of the milk. For the *quantitative* estimation of rennet activity, several methods are available. The enzyme solution should be carefully neutralized. The milk used should be free from fat and neutral in reaction. It may either be fresh or it can be kept for several days in an ice box if a small amount of chloroform is added. Milk from different animals differs in coagulability, woman's milk being the least coagulable. Some specimens of cow's milk are relatively incoagulable, especially if they have been boiled.[293] Dried milk powder can also be used, by dissolving 10 grams of powder in 100 cc. of distilled water at 50°C., then adding 0.5 cc. of a 20 per cent calcium chloride solution. Dried milk is preferable for comparative studies.

Most of the methods consist in determining the smallest amount of rennet which is required for the coagulation of a definite amount of milk in a definite period of time at a definite temperature. The same volume of different dilutions of the enzyme is added to a series of test tubes containing 10 cc. of milk. These tubes are placed in a thermostat, at the desired temperature, and examined at various intervals to determine in which tubes the coagulation of the milk has occurred. The tube containing the smallest amount of enzyme, in which coagulation has occurred, is used as the basis of calculation. If the amount of enzyme necessary to coagulate 1 cc. of milk in the given period of time is taken as a unit, and if, in a particular instance, 0.002 cc. of the enzyme solution is the minimum which will coagulate 10 cc. of milk then, $\frac{10}{0.002} = 5{,}000$ units.

In some methods, as in that of Morgenroth-Fuld,[465] the tubes containing the milk and the dilutions of enzyme are placed for twenty-four hours in an ice chest (or two hours at 16° to 17°C.[146]). The tubes are then placed in a water bath, at 37°C., for ten or fifteen minutes. The enzyme content of the preparation is calculated as outlined above. The activity of rennet may also be measured by means of an Ostwald viscosimeter; 1 cc. of the enzyme solution is added to 15 cc. of milk, the mixture incubated at 35°C. and its viscosity determined every two minutes.[758a] The product of the enzyme concencentration and the time required for the coagulation of milk is not a constant but may increase with an increase of enzyme concentration (see below). The reaction of the milk is of importance in this connection, the greater the acidity, the lower is the maximal value of that product.[498a]

Antirennet, a substance which inhibits the milk-coagulating action of rennet, is present in the serum of various animals, in urine and in woman's milk. It is more active against the rennet of animals than that of plants.[851] It can be demonstrated *qualitatively* by placing in each of two test tubes an amount of rennet sufficient to cause coagulation in ten to fifteen minutes and then adding 1 cc. of serum to one tube and 1 cc. of normal salt solution to the other. Both tubes are then kept at room temperature for ten minutes. Ten cubic centimeters of milk are then added to each and both tubes are placed in a water bath at 38°C. The milk in the tube containing the salt solution will coagulate in ten to fifteen minutes, but in the tube containing the serum, coagulation will not occur even after several hours. To estimate antirennet *quantitatively*, two identical series of tubes are prepared containing various dilutions of rennet. One cubic centimeter of normal saline is added to each tube of one series and 1 cc. of diluted serum to each tube of the other. After fifteen minutes, 5 cc. portions of milk are added to all of the tubes of both series, the contents shaken and the tubes incubated at 16° to 17°C. for two hours. They are next placed for ten minutes in a water bath at 37°C. and then cooled. The tube in each series containing the smallest amount of rennet in which coagulation has occurred is then determined. By comparing the two series, the strength of the antirennet action of the serum is demonstrated.[1298]

Rennet is probably a protease in character, as is indicated by the fact that it can also act to a limited extent upon proteins other than casein, i.e., legumin. The rennet of the stomachs of calves and other newly born animals is independent of pepsin, active at a weakly acid reaction, and digests only a few proteins; it may, however, change during the progress of development of the animal into pararennet or into normal pepsin,[999] which acts as a weak rennet.[923] The rennet of the stomachs of adult animals is often spoken of as *pararennet* (*parachymosin*), in contradistinction to the classical rennet of the calf's stomach. Pararennet is more sensitive than rennet to the effects of an alkaline reaction (alkalinity greater than pH 6.8 being very injurious[834]) and to high temperatures;[294] in a strongly acid medium, pararennet is more resistant to heat (75°C.) than rennet. The enzymes of the rennet and pararennet type also have the following differences:[1298] (1) In the case of rennet, the product of the enzyme concentration and the time of action is a constant; in the case of pararennet, the product increases rapidly with increases in the enzyme concentration; with a high dilution, the action of pararennet approaches zero. (2) The effect of calcium chloride on the activity of pararennet is greater than that on rennet.

The optimal temperature for rennet is 45°C., although it is also active at lower temperatures.[679] The destructive temperature depends upon the reaction (the enzyme being more resistant in alkaline solutions) and upon the concentration (less sensitive in higher concentrations) of the solution. When dried, rennet readily resists high temperatures. The optimal reaction is pH 6.0 to 6.5;[25,832,840] the enzyme is quickly destroyed at pH 9.0.

2. Hydrolytic Proteolytic Enzymes. The hydrolytic enzymes which digest proteins and their derivatives are divided[923] into: (1) *Proteases* which act upon native proteins, (2) *peptidases* which hydrolyze polypeptides to amino acids and (3) *amidases* which liberate ammonia from amino- and amido-compounds. A detailed study of the chemistry of the proteins and their derivatives may be obtained elsewhere.[15,1175] The processes of decomposition involve various enzymes and lead from the complex colloidal aggregates

(the proteins), through the polypeptides, to the crystalline amino acids (this last step being of primary importance in animal digestion) and finally to ammonia.

The *proteases* hydrolyze the proteins to albumoses and peptones; as, for example, pepsin of the stomach, pancreatic trypsin, certain tissue proteases and plant and microbial proteases. The fact that the two typical animal proteases, namely pepsin and trypsin act at distinctly different reactions, has led to the separation of the proteases into *pepsinases*, or those which have their optima at pH 2.0 to 5.0, and *tryptases*, with an optimum at pH 7.0 to 9.0. However, this separation does not hold true in the case of the plant and microbial proteases, as well as of certain tissue proteases, which may act both at a weakly acid and a weakly alkaline reaction.

The *peptidases* digest only albumoses, peptones, and other polypeptides, natural or synthetic. The erepsin of the intestine and of the pancreatic juice belongs to this group. The proteolytic enzymes of yeasts and other microörganisms, as well as of plant tissues, are usually peptidases.

The *amidases* finally complete the process of hydrolysis of the proteins, reducing the nitrogen to its simple inorganic form, namely ammonia. The action of this group of enzymes is of primary importance in the metabolism of plants and microörganisms, which seem to assimilate their nitrogen largely as ammonia and to synthesize their own amino acids.

a. Proteases. i. *Pepsin, propepsin and antipepsin.* Spallanzani in 1785 was the first to demonstrate the rôle of gastric juice in digestive processes, by performing experiments outside of the body.[1159] Schwann fifty years later pointed out that this digestion was due to the action of an enzyme, which he named pepsin.[1109] Pepsin is capable of hydrolyzing proteins, at an acid reaction, to proteoses and peptones. It is most active in the presence of hydrochloric acid, less so in the presence of other acids; it is very sensitive to alkalies. It is present in the gastric juice and gastric mucosa of almost all vertebrate animals, mostly in the fundus portion of the stomach and only to a slight extent in the cardia and pylorus. Pepsin is also found in the urine and to a more limited extent in the intestine. Various insectivorous plants produce a pepsin-like enzyme, though some of their proteolytic processes are due to bacteria. The pepsin of different animals varies both in its action upon different proteins and in its optimal reaction.

Preparation. Pepsin can be prepared either from the gastric juice obtained through a fistula in a dog's stomach or from the gastric mucosa. Pure gastric juice (from a dog's stomach) can be purified by filtration and dialysis for twenty hours against distilled water, at a low temperature. The turbid liquid is then centrifuged, the residue placed upon a filter, washed with a little distilled water and dried. In this way, colorless spherical particles of the enzyme are obtained. The enzyme that remains in the supernatant fluid of the centrifuge tube can be recovered by precipitation with half saturated ammonium sulfate. The precipitate is filtered, dialyzed, dissolved in 0.2 per cent hydrochloric acid, precipitated by dialysis, filtered and dried. To prepare pepsin from the gastric mucosa, the fundus portions of ten swine stomachs are macerated and extracted for five days with six liters of 0.5 per cent HCl at 37°C.[960] The liquid is filtered through a thick layer of filter paper, then placed in parchment dialyzers and dialyzed in flowing tap water for twenty-four hours. The precipitate which forms is separated by centrifugalization, digested for one hour, at 37°C., with 30 to 40 cc. of 0.2 per cent HCl and then filtered. The filtrate is dialyzed in distilled water for fifteen to twenty hours. The resulting precipitate is again extracted and dialyzed. The final precipitate is dried over sulfuric acid. To recover the pepsin from the solution left after the first centrifugalization, the solution is treated with basic lead acetate and ammonia. The precipitate which is formed is filtered off and treated with a saturated solution of oxalic acid and again filtered. The acid filtrate is dialyzed in tap water twenty-four to thirty-six hours; the resulting precipitate is filtered off, extracted in acid and dialyzed in distilled water; the process may be repeated. The residue is finally dried over sulfuric acid. In a pure state, pepsin is a white or yellowish amorphous powder, soluble in water, salt solution, dilute acids and glycerin, it is precipitable from solutions by ammonium salts and alcohol and is readily adsorbed by basic substances.

Measurement. Qualitatively the presence of pepsin can be demonstrated by its action upon coagulated fibrin, coagulated egg-albumin or elastin. The enzyme solution must be acid (0.1 per cent HCl, or pH 2.0). The carmin-fibrin of Grützner[504] may also be used; the solution of the fibrin by the pepsin is accompanied by an increase in the color intensity of the liquid. For the *quantitative* determination of the action of pepsin, various methods are available. These are usually based upon one of two principles—either the determination of the amount of protein digested at the end of a given time or the determination of the time required to effect a given change in the protein. The latter is fundamentally more satisfactory, for reasons which will have already been advanced (see p. 168), especially if several determinations are made at intervals during the course of the protein digestion. The estimation of the amount of protein digested may be done in several ways: (1) The measurement of the amount of an insoluble (solid) protein which remains unliquefied after the enzyme has acted upon it; (2) the titration of a solution or suspension of protein to determine the amount which remains undigested; (3) the titration of the decomposition or digestion products (peptones, amino acids, etc.) which have been formed by the action of the enzyme upon a protein or polypeptide and (4), the course of the digestion of the protein by the enzyme may be studied by determining at intervals the changes in the physical or physico-chemical condition of the protein solution (viscosity, conductivity, turbidity, etc.). A few of the methods are described here in detail; others are given elsewhere.[1071] These same methods may also be adapted to the study of the action of any proteolytic enzyme by merely adjusting the reaction of the substrate to the optimum for the particular enzyme employed.

Mett method.[814,1128] Glass tubing, 2.5 to 3 mm. in diameter, is cut into 20 cm. lengths, carefully cleaned and the ends drawn out into capillaries. The white of an egg is cut into pieces, filtered through cheese cloth and placed in a vacuum for one hour to prevent the subsequent formation of air bubbles. The egg-white is then aspirated into the prepared tubes and the ends of the latter sealed by fusing; the egg-white is coagulated by placing the tubes for fifteen minutes in water heated to 95°C. The tubes are then cooled slowly. To perform the test, 20 cc. portions of a solution of pepsin in 0.05 N HCl are placed in flat bottomed containers, of such a size that the depth of the solution is about 1 cm. The tubes of the coagulated egg-white are cut into 2 cm. lengths with squared ends, 2 pieces are placed in the enzyme solution and the container placed in a thermostat at 40°C. At the end of twenty hours' digestion, the capillary tubes are taken out and the digested column measured microscopically. The method cannot be used for accurate quantitative comparisons. The use of *carmin-fibrin or fuchsin-fibrin,* whereby the action of the enzyme is measured by the increase in the color of the solution due to the decomposition of the fibrin is also useful.[504,1187,1298] A clinical test for pepsin consists in measuring the volume of the starch freed from spaghetti during digestion in the stomach.[149]

The determination of the amount of undigested substrate can be carried out as follows:[1128] A 2 per cent casein solution is prepared by dissolving casein in 0.05 N HCl (for pepsin) or in 0.1 N NaOH solution (for trypsin). This protein solution is warmed to 40°C., the enzyme solution added, and the mixture incubated. At the end of the incubation, 100 cc. of the digestion mixture are added to 25 cc. of 20 per cent Na_2SO_4 (plus 5 cc. N/2 HCl, if the digestion mixture is alkaline) and, after standing one hour, the resulting precipitate is filtered off. The action of the enzyme can be measured either by determining the residual casein (by determining the total nitrogen of the precipitate) or by analyzing the filtrate by one of the methods described below.

The *edestin method* of Fuld and Levison[366,466] is based upon the fact that the addition of sodium chloride to an acid solution of edestin precipitates the undigested edestin, but not the digestion products. One-tenth gram of edestin is dissolved in 100 cc. of 0.03 N HCl by warming to boiling, and filtering if necessary. Various amounts of the enzyme solution are placed in a series of tubes, using 0.03 N HCl as the diluent; 2 cc. of the cooled edestin solution is added to each tube; these are then kept for thirty minutes at room temperature. One-half cubic centimeter portions of 33 per cent NaCl are then added to all of the tubes and the tube containing the smallest amount of enzyme in which no turbidity is produced (indicating complete protein digestion) is taken as the limit. From this dilution, the pepsin concentration is determined, using as a unit the amount of enzyme which will completely digest 1 cc. of edestin solution in thirty minutes at room temperature. By this method the undiluted gastric juice of dogs obtained by Pavlov's method contains 400 to 1600 units. Casein and glacial acid may be substituted for the edestin and sodium chloride in this method.[611,1161]

The activity of the enzyme can also be determined by measuring *the products of digestion*, either in the filtrate, obtained as described above, or directly in the digestion mixture. If the total nitrogen of the digested protein is used as a measure, the undigested protein should first be filtered off. The increase in amino nitrogen, which results from the hydrolysis of the protein and its cleavage products, is also a quantitative index of enzyme activity and can be determined by one of several methods.[202,451,1157,1212]

The method of Volhard and Löhlein[1232] is based upon the fact that casein combines with a definite amount of hydrochloric acid and, if the casein has been digested by the enzyme, the acid is liberated. The increase in the amount of free HCl, compared with that of the original solution (determined by titration) serves as a measure of enzyme action. A 5 per cent casein solution is prepared by suspending the casein in a small amount of water, dissolving in NaOH solution (using 8 cc. N NaOH for 10 grams of casein) making up to volume with water and warming to 85° or 90°C. One hundred and fifty cubic centimeters of N/14 HCl and 100 cc. of the casein solution are mixed, the mixture warmed to 40°C., the proper amount of pepsin solution added and the volume made 300 cc. with water warmed to 40°C. The mixture is then placed in a thermostat at 40°C. At the end of the incubation period, 100 cc. of 20 per cent Na_2SO_4 are added, thus precipitating the undigested casein; the solution is filtered and 100 cc. of the filtrate titrated with N/10 NaOH, using phenolphthalein as an indicator. The difference between the titration value of the digest and that of the original solution and of the pepsin solution indicates the increase in the acidity of the digest. If v is the increase in acidity, t the time in hours, f the quantity of enzyme used, and x the actual pepsin concentration,

$$x = \frac{(f \times t)^2}{(v^2)}, \text{ according to the Schütz-Borrissov rule}^{1108}$$

Among the physical and physico-chemical methods used for determining the action of pepsin and other proteolytic enzymes, the following should be mentioned: *the refractometric method*[1056,1100] (see p. 210), the *nephelometric method*[1041] and the *viscometric method.*[900] The last method, in our experience, is the most accurate as well as the simplest. It is fully described on page 210 in connection with the measurement of trypsin.

Influence of environmental conditions. The optimal reaction for pepsin is pH 1.8 to 2.2, depending upon the isoelectric point of the protein, with all acids except acetic. At pH 4.0, its activity is very slight.[895] Hydroxyl ions have a strongly inactivating effect while proteins have a protective action. Strong alkalies destroy the enzyme completely. On the acid side of the optimal hydrogen ion concentration and at the optimum, the ions Na^-, K^-, Ca^{--}, Al^{---}, Cl^-, NO_3^-, SO_4^{--} do not exert any measurable injurious action in weak concentrations, but cause injury in high concentrations; on the alkaline side of the optimum, low concentrations of these ions have a stimulating effect which changes to an injurious one with an increase in concentration. All of the above ions act qualitatively alike but quantitatively different.[1041]

The optimal temperature for the action of pepsin is 38° to 40°C., and its activity rapidly diminishes at 50° to 65°C. In a dry condition, it can resist even 100°C. Twenty per cent alcohol has an injurious effect[515] upon pepsin; chloroform and toluol injure only in high concentrations. Formaldehyde is not inhibitory in concentrations of even 5 to 10 per cent.[623] A high concentration of sugar reduces the activity of pepsin. Blood, probably because of its antipepsin content, inhibits pepsin.[566] Depending upon the quantity present, lecithin exerts an inhibiting or accelerating influence on the action of pepsin;[698] cholesterol is injurious even in 0.5 per cent concentration.[336]

Propepsin, a true zymogen, has been described in the gastric mucosa and urine.[477] It is supposedly activated by hydrochloric acid. However, the existence of this zymogen is doubted.

Antipepsin does not completely arrest the action of pepsin but weakens it.[1059] It is found in the gastric juice and mucosa, in the intestinal wall, in blood serum, oedema fluids and extracts of microörganisms. The existence and strength of the antipepsin can be determined by comparing, by one of the methods described above, the action of two specimens of pepsin, one of which has been treated for thirty minutes, at room temperature, with the preparation suspected of containing antipepsin.[375]

Although the synthesizing action of pepsin, or *plastein formation* has not as yet been definitely established, the formation of a flaky precipitate has been observed in a clear solution of peptones to which rennet,[295] papain[695] or proteases of different origin[999] have been added. Proteins can probably be synthesized by enzymes; the process is reversible, the equilibrium point depending upon the reaction and the concentration of the substrate; concentrated solutions, weak acidity (pH 4.0) and higher temperature favoring synthesis.[1052]

ii. *Trypsin, trypsinogen, enterokinase, antitrypsin.* Trypsin is the proteolytic enzyme of the pancreas which is secreted into the lumen of the intestine in the form of a zymogen.[694] This enzyme converts nearly all proteins into amino acids. Its activity is greatest in alkaline solutions, the optimum varying for different preparations. The optimal reaction for animal trypsin is pH 8.0 to 9.0[751] with fibrin as a substrate, and pH 6.0 to 6.5 with casein; with gelatin, the optimum is pH 8.0. It is inactive at pH 4.0, but not destroyed and can be kept with the least deterioration at pH 5.0.[899] Its maximal activity is at pH 11.5, but it is rapidly destroyed at this reaction. The rate at which pepsin or trypsin digests gelatin, casein and hemoglobin is at a minimum at the isoelectric point of the protein and at a maximum at that pH at which the protein is completely combined with acid or alkali to form a salt.[899] Trypsin and pepsin behave like univalent ions, the former being a cation and the latter an anion; trypsin reacts with the anion of the protein molecule and pepsin with the cation of the protein.[901,904] The physical properties of the protein solution have little or no effect upon the rate of digestion. The optimal temperature for trypsin, at an optimal reaction, is about 60°C., although at this temperaure it is rapidly destroyed. At 37° to 39°C., the enzyme is much more resistant. The destructive temperature is 75° to 80°C.

The action of the enzyme is inhibited by the products of hydrolysis.[902] Immune serum and complement alter typhoid and colon bacilli so that they are more readily digested by trypsin.[617] The bacillus of Friedlander or red blood cells exposed to the action of fresh serum and then washed by centrifugalization are digested by trypsin or by leucoprotease but serum heated to 60°C. has no such effect.[337] It has been reported that, if trypsin is allowed to act upon hemoglobin in the presence of dextrose, bilirubin is formed,[284,573] but this work could not be confirmed.[1017]

Trypsinogen and enterokinase. Trypsin is present in the pancreas in an inactive condition (trypsinogen) and is activated by a special kinase, enterokinase, which originates from the intestinal epithelium. The activity of pancreatic juice may perhaps be associated with a colloidal protective substance, which is responsible for the characteristic properties of trypsin, namely, its proteolytic power and lack of resistance to heat, acids, and alkalies, while enterokinase is an enzyme capable of acting only on the complex of trypsinogen and its protective colloidal covering and initiates the reaction which is then completed by the liberated trypsin. According to this view the activation of trypsinogen is essentially an autocatalytic process.[1118] Enterokinase may be regarded as a catalyst (it is destroyed at 50°C.) which forms, out of trypsinogen, a new substance, trypsin,[104] or as a co-enzyme, the presence of which is necessary to activate the trypsin,[1245] or the action of enterokinase upon trypsin may be in the nature of an activation similar to that of hydrocyanic acid upon papain. The concentration of kinase can be greatly increased by selective precipitation and adsorption;[1245] by adsorption with aluminium hydroxide in alkaline solutions, the kinase can be removed quantitatively from the active enzyme.

Preparation. Trypsin can be obtained by duodenal tube, from a fistula or from the pancreatic gland itself. When pancreatic extract is precipitated with alcohol, a product known commercially as *pancreatin* is formed which contains the trypsin. By dissolving the enzyme is 0.03 per cent ammonia and precipitating successively with acetic acid, then redissolving with sodium carbonate solution, trypsin can be purified. Trypsin can also be extracted with 40 per cent alcohol and precipitated with alcohol-ether. The enzyme can also be precipitated by a combination of sodium chloride and ammonium sulfate at 40°C.[791] By methods of selective adsorption, the proteolytic enzymes of the pancreatic juice can be separated into trypsin and erepsin, the latter being identical with intestinal erepsin. Trypsin in itself is inactive towards synthetic peptides; it is the erepsin of the pancreatic juice which enables it to hydrolyze the peptides. Before activation, trypsin will hydrolyze certain peptones, protamines and histones, but not proteins; on activation with enterokinase, trypsin will also digest native proteins.[1245a] Further information on the purification of trypsin may be obtained elsewhere.[825,1283]

Measurement. The action of trypsin can be demonstrated *qualitatively* by the use of coagulated fibrin colored with congo red or with diphenylrosanaline in alkaline solution;[1153] the digestion of the fibrin is accompanied by an increase in the color of the solution as compared with that of the control. This method can also be utilized for *quantitative* purposes. The Mett method[814,1128] described on page 203 can also be employed for measuring the action of trypsin by substituting sodium bicarbonate solution for hydrochloric acid. The increase in soluble nitrogen, or amino acid nitrogen[202, 451, 1157, 1212] can also be used for measuring tryptic activity. If the Volhard-Löhlein method (p. 205) is used, the 150 cc. of N/14 HCl should be added not before, but after the digestion is completed, during precipitation with Na_2SO_4. Several other methods have been advocated.

Willstätter titration method:[1289b] Ten milligrams of the enzyme powder are dissolved in 1.2 cc. of water and activated for thirty minutes, at 37°C., with 0.3 cc. kinase solution (prepared by treating 5 grams of dried intestinal mucosa with 250 cc. of N/20 NH_4OH for two hours at 30°C., then concentrating to one-half volume). The mixture is made up to 5 cc. with water and placed in a flask; 2 cc. of a N NH_3-NH_4Cl mixture (1:2) are added to serve as a buffer (equivalent to pH 8.9, and, in the presence of the gelatin, to pH 8.0); 5 cc. of warm 15 per cent gelatin solution are then added, making the total volume 12 cc. This mixture is incubated for exactly twenty minutes. At the end of that period, the reaction is stopped by pouring the digestion mixture into 55 cc. of boiling absolute alcohol. The flask is washed with another 55 cc. of hot alcohol, which has been made alkaline to thymolphthalein (0.5 cc. of a 0.5 per cent solution) by the addition of a few drops of 0.2 N KOH solution. The mixture is then titrated with 0.2 N alcoholic KOH solution until a gray-green to blue-green color is obtained. A unit of trypsin by this method is that amount of enzyme which will increase, under these conditions, the acidity of the mixture an amount equivalent to 2 cc. of 0.2 N KOH solution.

The Fuld-Gross method[466,500] is based upon the precipitation of undigested casein in an acetic acid alcohol solution, in which the products of digestion are not precipitated. Casein (Hammarsten) is dissolved in N NaOH solution, heated to the boiling point, adjusted to a proper reaction, and diluted to a concentration of 0.1 per cent. The enzyme solution is placed in a series of tubes, in increasing dilutions with distilled water. Two cubic centimeter portions of the casein solution are added to the tubes, which are then placed in a thermostat, at 38°C., for one hour. The tubes are cooled and six drops of acetic acid alcohol (1 cc. acetic acid, 50 cc. water and 50 cc. of 96 per cent alcohol) are added to each tube. Those tubes, in which undigested casein is still present, become turbid, while the tubes in which the casein has been completely hydrolyzed remain clear. The lowest concentration of enzyme which completely digests the casein is taken as the basis for the calculation of the number of trypsin units contained. One trypsin unit, by this method, is that amount of enzyme which will hydrolyze completely, in one hour, 2 cc. of the casein solution, i.e., if 1 cc. of a 1:100 dilution of the enzyme solution is the lowest concentration which has completely digested the 2 cc. of casein, then the enzyme is said to contain 100 trypsin units per cubic centimeter.

Increase in electrical conductivity.[105] The course of casein decomposition by trypsin can be conveniently studied by the use of the conductivity method, since the hydrolysis of a protein results in the formation of substances possessing a higher conductivity than that of the original protein. The solutions are placed in conductivity cells having electrodes about 1 cm. square and about 1 cm. apart.

Refractometer method. In the presence of a definite amount of alkali, 1 gram of casein has the same refractometric index as 1 gram of digested casein, so by precipitating the undigested casein out of solution, the refractive index of the remaining digested casein can be determined.[1028] The enzyme solution is added to 100 cc. of 2 per cent casein (dissolved in 16 cc. N/10 NaOH) and incubated at 37°C. At definite intervals of time, 10 cc. of the mixture are treated with 10 cc. of N/40 acetic acid, to precipitate the undigested casein, and the refraction of the filtrate is then measured. The refractive index of an equal amount of acetic acid and of the amount of NaOH solution used to dissolve the casein is also determined and subtracted from the first value. The difference indicates the refractive index of the digested casein.

Optical methods. Optically active di-, tri- or polypeptides have often been used for the study of proteolysis. The hydrolysis of the substrate results in a change in the polariscopic rotation of the substrate. The method is very rapid and convenient and permits the study of small amounts of enzyme; but the fact that the change in rotation does not proceed progressively with the amount of enzyme or the time of enzyme action interferes with the use of the method for quantitative comparisons.[636,1128]

Viscometric method.[499,900] This is one of the best methods for the quantitative determination of trypsin and pepsin. The substrate is prepared by placing 200 grams of Peter Cooper's granulated gelatin in an enameled bucket containing 6000 cc. of N/128 acetic acid in order to wash it at its isoelectric point, pH 4.7.[740] A few lumps of ice are placed in the fluid to keep the temperature at 3°C. The suspension is stirred 4 or 5 times during the next half hour and then allowed to settle. The supernatant fluid is decanted. The whole process is then repeated. After the second washing, the semi-solid gelatin is poured on a large Buchner filter over which one thickness of muslin has been spread. The excess fluid is removed by suction filtration. The solid gelatin is then placed in a flask and melted at 50°C. Ten cubic centimeters of this melted gelatin should be completely dried in an oven at 100°C. for twenty-four hours and the residue weighed. Ten cubic centimeters of gelatin prepared in this way usually weigh 1.6 grams when thoroughly dried. For trypsin digestion the concentrated gelatin preparation is diluted with M/10 Na_2HPO_4 so as to contain 3 grams of gelatin by dry weight per 100 cc., then its reaction is adjusted to pH 7.4 with M/10 H_3PO_4. (For pepsin digestion the concentrated gelatin is diluted to 3 per cent and its reaction adjusted to pH 3.0 with dilute phosphoric acid.) The diluted gelatin is distributed in 250 cc. amounts in stoppered Erlenmyer flasks, a small amount of thymol added to each flask as a preservative, and if stored in a refrigerator at 3°C. can be kept satisfactorily for a year or more. The viscosimeters used in this method are made after the pattern of the Ostwald type and are calibrated by regulating the time of outflow of 5 cc. of water at 34°C. to be sixty seconds between two marks. The gelatin-water time ratio is approximately 3.

For the determination of tryptic activity, 10 cc. of this 3 per cent gelatin at pH 7.4, after it has been melted by being kept at 34°C. overnight, are placed in each of two Ostwald viscosimeters immersed in a glass water bath at 34°C. and the time required for 5 cc. of the gelatin to flow through the capillary tube determined several times with a stop watch. (This time will hereinafter be designated as the viscosity of the substrate.) Five-hundredths to 0.4 cc. of the preparation containing trypsin are then added to one of the viscosimeters and the same amount of the enzyme preparation, after inactivation by boiling, to the other as a control. The contents are then thoroughly mixed by shaking and the time noted. The viscosity of these mixtures is determined at intervals of three or more minutes for a period of one to two hours. (The determination of peptic activity is similar, except that 3 per cent gelatin at pH 3.0 is used.)

The results obtained are plotted on coördinate paper, as described on page 167 in connection with the viscometric measurement of amylase. A unit of trypsin has been defined as that amount of enzyme which requires sixty minutes to reduce the initial viscosity 20 per cent. If 0.1 cc. of an enzyme preparation requires sixty minutes to reduce the initial viscosity 20 per cent in sixty minutes, 0.2 cc. will reduce it a similar amount in thirty minutes. If the initial viscosity is reduced 20 per cent in ten minutes, the enzyme preparation is assumed to contain $\frac{60}{10}$ or 6 units, etc.

Antitrypsin injures the action of trypsin and is destroyed at 64°C.

It is present in blood serum, exudates and transudates, organ extracts and in almost all protein-containing solutions; the amount in the serum of animals can be increased by the injection of sterile pancreas solutions. The usual method of demonstrating the action of antitrypsin is by placing equal quantities of an active trypsin solution in two tubes. To one tube, a portion of the antitrypsin solution (blood serum, etc.) is added and to the other a corresponding amount of the antitrypsin solvent (salt solution, when serum is used as a source of antitrypsin); the mixtures are allowed to stand fifteen minutes. Colored fibrin flakes or casein solution are added to the tubes, which are then incubated at 37°C. The decomposition of the protein is found to proceed rapidly in the control but only very slowly in the tube containing the antitrypsin. By modifying one of the quantitative methods described above, the action of antitrypsin can be measured quantitatively. The variation of the antitryptic activity of the blood in various diseases and conditions has been discussed in a previous chapter on the enzymes of blood serum (see p. 77).

iii. *Nucleases.* This group of enzymes which is present in the plant and animal kingdom gradually reduces the complex nucleins and nucleic acids to their simpler constituents. The original idea that nuclease was a single enzyme is no longer tenable. At least three groups of enzymes are concerned in this process:[722,724] (1) the *nucleinases*, which liberate the free nucleotides from the nucleic acids; (2) the *nucleotidases* (esterases), which hydrolyze the ester structure of the nucleotides, liberating nucleosides (glucosides) and phosphoric acid; (3) the *nucleosidases* (glucosidases), which hydrolyze the glucoside, liberating the carbohydrate and purine bases. A description of the last two groups of enzymes has been given elsewhere (see pp. 148 and 189). In addition to these three groups, there is probably a fourth group of enzymes, proteolytic in nature, which converts nucleoprotein into proteins and nucleic acid.

Enzymes which are capable of hydrolyzing nucleins and nucleic acids are found in the pancreas and small intestine of dogs and other animals, in the thymus and nerves of calves and other animals, and in various plants and microörganisms. For demonstrating the presence of an enzyme capable of liquefying nucleic acids, a 4 per cent solution of the sodium salt of α-thymonucleic acid may be used; it is a liquid when warm, and a solid at room temperature.[1069] A warm solution of this salt is treated with the enzyme, incubated for twenty-four hours and then cooled; the degree of liquefaction is an index of enzyme activity. To demonstrate whether any of the nucleic acid remains unchanged, the mixture is acidified with acetic acid and filtered while hot. A 5 per cent alcoholic solution of sodium acetate is then added to the cool filtrate until no further precipitate is formed. This precipitate is dissolved in a small volume of hot water and if gelatinization occurs when the solution is cooled, some of the original nucleic acid still remains undigested. The optical methods can also be employed for measuring the activity of the nucleases.[922a]

b. Peptidases. i. *Erepsin and ereptases.* Erepsin is the enzyme which converts albumoses, peptones and simpler polypeptides, but not native proteins (except casein) into amino acids.[263] It acts only at a reaction of about pH 8.0.[825] Erepsin is found in intestinal and pancreatic juices, and in extracts of nearly all animal organs, in numerous plants and microörganisms. Animal erepsin is prepared by grinding the scrapings of the intestinal mucosa with sand. This mass is then extracted, for three days, with 0.1 per cent sodium carbonate, in the presence of toluol or chloroform, and frequently shaken.[1016] The mixture is then pressed through cheese cloth and filtered through folded filter paper. Sufficient acetic acid is then added to the filtrate to make the reaction pH 5.2, and the liquid again filtered. The clear filtrate is then dialyzed. The mass (ground with sand) may also be extracted with glycerin for three days.[390] This extract is then acidified with acetic acid, filtered and the clear filtrate dialyzed for five days. The clear solution which does not contain any inorganic salts or amino acids is then diluted to the volume of the original mass of intestinal mucosa. The enzyme may be kept in this soluble form.[212] Erepsin can also be extracted from fresh intestinal mucous membrane with 87 per cent glycerol. The solution is diluted with water and the enzyme is precipitated by various reagents, dilute acetic acid giving the highest recovery. The enzyme is then adsorbed from the aqueous solution by aluminium hydroxide at pH 4.7. It can be redissolved from the adsorbate by means of an alkaline phosphate solution, erepsin being thus separated from the trypsin.[1245a]

Yeast ereptase is prepared by grinding 500 grams of washed, pressed beer yeast with 30 grams of calcium carbonate and 30 cc. of chloroform in a porcelain dish; the mass is allowed to autolyze for three days at room temperature and then pressed through cloth and filtered through folded filter paper.[672] The clear liquid is then dialyzed for six days, under diminished pressure in the presence of toluol. The liquid is then filtered and the filtrate kept as a source of the enzyme.

For measuring the action of erepsin, methods based upon the disappearance of the biuret reaction of a peptone solution[363] or upon the change in the precipitate, produced by phosphotungstic acid, were formerly used.[1298] However, the increase in the content of amino-acid nitrogen[202, 451, 1157, 1212] or in the electrometric conductivity of a peptone or casein solution to which the enzyme has been added, is a more convenient method for estimating the activity of ereptase.[458,1024] Erepsin can also be measured by titrating with an alkali (in 85 per cent methyl alcohol) the acidic groups liberated during its action upon glycyl-glycin.

The ereptase of yeast is much less sensitive to the influence of salts (0.5 N solutions are not injurious) than the erepsin of the intestine (0.02 N solutions of salts are injurious).[313] The optimal temperature for animal erepsin is 45 to 50°C.; for yeast ereptase 55°C. the optimal reaction is at pH 7.8. The hydrolysis of peptides by erepsin follows the course of a monomolecular reaction.[1245a]

ii. *Peptolytic enzymes.* Peptolytic enzymes hydrolyze simple peptides to their constituent amino acids; they are often believed to be identical with the ereptases and are found throughout the animal body, in many plant tissues and in microörganisms. Their optimal reaction is usually pH 7.8;[313] it depends upon the nature of the substrate, being pH 6.24 for a complex polypeptide and pH 8.5 for glycyl-*l*-leucine.[11] Different extracts are capable of hydrolyzing different peptides. For qualitative and quantitative studies, 0.4 N glycyl-*l*-glycin may be used with a 0.04 M phosphate mixture at pH 8.5 as a buffer solution. The increase in amino nitrogen which results from the hydrolytic action of the enzyme is usually used as an index of peptolytic acitivity; changes in polarization can also be employed as a measure of enzyme action.[1298] A detailed study of the peptidases of man and animals may be obtained elsewhere.[968]

c. Amidases. i. *Desaminases* (*aminoacidases*). The enzymes which liberate ammonia from amino acids, namely the *desaminases* (*aminoacidases*) should be differentiated from the *desamidases* (*amidases*), which act upon acid amides.[226] The latter can be demonstrated in the mycelium of *Asp. niger* and are able to hydrolyze the amide group of the asparagine molecule. Desaminases act only in the presence of methylene blue and produce both ammonia and carbon dioxide in the ratio of $\frac{NH_3}{CO_2} = \frac{14}{44}$. This corresponds to the reaction of oxidative deaminization:

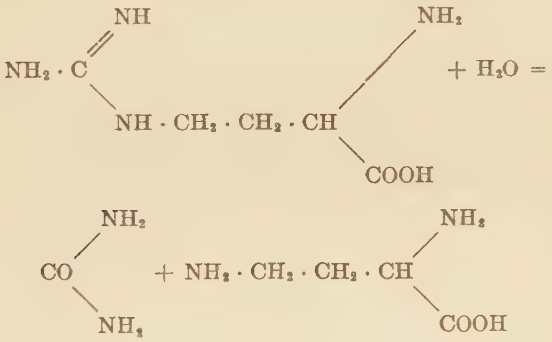
(1) $R \cdot CH \cdot NH_2 \cdot COOH + O = R \cdot COH + NH_3 + CO_2$

The amino group is thus broken off from the amino compound not only through hydrolytic processes, but also by oxidation and even by reduction:

(2) $R \cdot CH \cdot NH_2 \cdot COOH + H_2O = R \cdot CH \cdot OH \cdot COOH + NH_3$
(3) $R \cdot CH \cdot NH_2\ COOH + H_2 = R \cdot CH_2 \cdot COOH + NH_3$

The only definite aminoacidase or desaminase thus far known is tyrosinase, described under the oxidases (see p. 237); very little is known of the enzymatic nature of the deamination of the other amino acids.

ii. *Arginase.* Arginase forms ornithin and urea from arginine:[685]



The enzyme acts only upon *d*-arginine, not upon the laevo-form. Liver arginase may also hydrolyze *d-l*-arginine.[427] Arginase is found in the liver, nerves, thymus, muscles and intestinal wall of various animals, especially in the liver and kidney of mammals, the liver of all fishes, and kidneys of fishes and birds; rarely in the tissues of invertebrates.[589] It is also abundantly distributed in plants[661] and is found in yeasts,[1127] in fungi,[609] and in dead cultures of certain bacteria.[564] The enzyme is found much more abundantly in males of different animals than in females.[357]

The enzyme is prepared in the following manner:[685] The liver of a dog, killed by bleeding, is macerated in a meat grinder and then thoroughly mixed with infusorial earth and pressed until 45 per cent of the liver is obtained in the pressed juice. A mixture of 2 volumes of alcohol and 1 of ether is then added to the juice. The resulting precipitate is filtered, washed with ether and dried in vacuo over sulfuric acid. Five grams of powder, only partially soluble in water, are thus obtained from 200 grams of liver. One-tenth gram of this powder is capable of decomposing completely 2 grams of arginine in twenty-four hours. The enzyme is stable in dry and liquid preparations. The enzyme may also be extracted from the liver by digestion in an incubator, for twelve to twenty hours, with 0.2 per cent acetic acid solution. The solution is filtered and precipitated with alcohol-ether or ammonium sulfate. The precipitate is freed from the salt by dialysis.

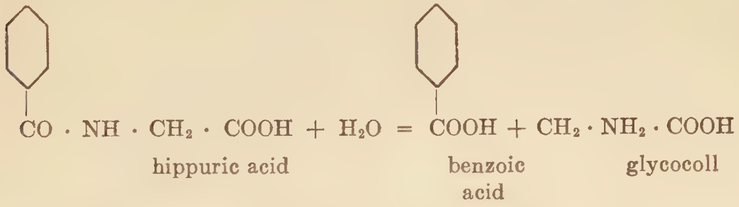
To test the strength of the enzyme, 1 to 2 grams of arginine or arginine carbonate are dissolved in water. A suspension of 0.5 to 1.0 gram of the above powder in 50 cc. of water or 20 to 25 cc. of the liver extract and some toluol are then added to the arginine solution and the mixture incubated for five to ten days. At the end of the incubation period, phosphotungstic acid is added, the solution filtered and the total nitrogen determined in the filtrate. If the liver extract is used, the protein should first be coagulated by boiling and the total nitrogen in the solution determined before and after precipitation with phosphotungstic acid. The amount of total nitrogen in the filtrate serves as an index of the amount of urea formed. The precipitate is treated with barium hydroxide and the solution filtered. The filtrate is acidulated with sulfuric acid and the arginine precipitated with silver sulfate and barium hydroxide. The nitrogen content of the silver precipitate indicates the amount of undecomposed arginine, while the nitrogen content of the filtrate from the silver precipitate indicates the amount of ornithin formed. As a control, the same process is carried out with the enzyme solution without the arginine. The determination of amino nitrogen can also be used as a measure of the action of the enzyme, since arginine has only one and ornithin has two free NH_2 groups.[359] The arginase unit has been defined as the amount of enzyme which will produce, in a mixture of 10 cc. of a 1 per cent arginine solution and 5 cc. of buffer mixture (glycocoll—NaOH—NaCl) of pH 9.5, in sixty minutes, at 38°C., sufficient urea to produce 0.34 mgm. of ammonia, when treated with urease.[357]

The optimal reaction for arginase is pH 7.0 and the optimal temperature 50°C.; at this reaction and temperature 92.2 per cent of the theoretical yield of urea may be obtained.[588] There is some evidence, however, that the optimal reaction for arginase is pH 9.5 to 9.8.[357]

iii. *Desamidases.* These enzymes which can act upon various acid amides (acetamide, oxamide, benzamide, etc.), as well as upon the amide asparagine, have been found in various animal tissues and in plants.[661] The formation of ammonia in plants is a result of the action of desamidases.[224–226] These enzymes are also formed by fungi,[339,1136] yeasts and placental tissue.[774]

An *asparaginase* has been demonstrated in the blood serum of the guinea pig, the liver of the albino rat, pig and guinea pig, and the liver and kidney of certain birds.[260] The enzyme was also found in practically all mammalian herbivora and in certain organs of omnivora (except in man and carnivora). The enzyme is formed as a specific reaction in response to the presence of asparagine in the food.

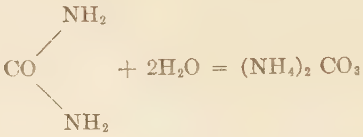
Histozyme belongs to the desamidase group; it acts upon hippuric acid and its homologues, such as *d*-α-benzoyl-amino-butyric acid, *l*-benzoyl-leucine and benzoyl-α-alanin, but not upon benzoyl-β-alanin and others.[1152]



Histozyme is found in nerves, various animal organs, as well as in fungi (*Asp. oryzae*)[888] and various bacteria. The enzyme can be prepared from organs by maceration and extraction with water or by the Buchner pressure method. In the case of fungi, acetone-ether preparations can be used. The method of demonstrating the action of the enzyme is based upon the extraction of the benzoic acid with ligroin. On removing the ligroin, the typical crystals of benzoic acid remain.[1298]

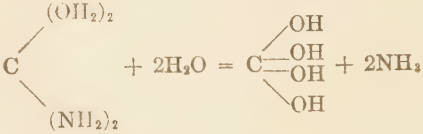
iv. *Urease.* Urease is the most important and most widely distributed desamidase; it hydrolyzes urea to ammonia and carbon dioxide and is formed by various bacteria,[1223] fungi,[483,1136] several plant organs and tissues, especially seed,[599] and is also found in putrid urine. The presence of leucine and one other amino acid is required for the formation of urease by urea bacteria and *B. proteus.*[605] Urease is an endo-enzyme and cannot be easily separated from the bacterial cells. An active preparation, which will keep for several years can be obtained by adding alcohol to a culture, or the dry colonies can be extracted with a buffer mixture at pH 8.9.[1185] The preparation of soy bean urease consists in removing the fat from finely ground seed with petroleum ether, extracting with water and concentrating by evaporation or by precipitation with alcohol, whereby the product becomes insoluble in water, or with acetone which tends to injure the enzyme.[586,604,1011,1213] Urease can also be prepared by extracting 200 grams of soy bean meal with 800 cc. of H_2O and 200 cc. of 95 per cent alcohol, for twelve hours. The resulting milky liquid is then poured off and is precipitated with 800 cc. of 95 per cent alcohol. The precipitate is filtered off and ground slowly in a mortar with 100 cc. of H_2O. This extract is filtered after two hours. Ninety cubic centimeters of the filtrate are precipitated with three volumes of absolute alcohol. The precipitate is allowed to settle; this process can be repeated several times. The final precipitate is dried in vacuo. *Gastric urease* has been obtained from the mucosa of the stomachs of dogs, by grinding it with sand, drying in vacuo and extracting with water.[736] This enzyme is probably identical with soy bean urease. A three to five week old culture of *Asp. niger* grown in peptone-sugar solution, freed from spores and treated with acetone and ether, can be used as a source of urease.[1136]

There are several theories in regard to the manner in which urea is decomposed. Dumas, in 1830, suggested that it followed the formula:



$$CO(NH_2)_2 + 2H_2O = (NH_4)_2\,CO_3$$

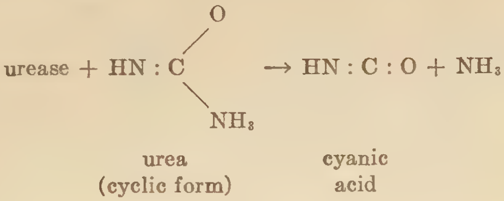
Later the process was considered to occur as follows:[45]



$$C(OH_2)_2(NH_2)_2 + 2H_2O = C(OH)_4 + 2NH_3$$

According to another hypothesis, urea decomposition takes place in two different stages, with the formation of ammonium carbonate as the intermediate product.[1309a] It has been suggested that urease is a dissociating enzyme and that the neutral urea molecule is decomposed into an alkaline component, ammonia, and an acid component, cyanic acid, which is rapidly hydrolyzed into NH_3 and CO_2:[426]

1. Dissociation stage:



$$\text{urease} + HN : C\begin{matrix} \diagup O \\ \diagdown NH_3 \end{matrix} \longrightarrow HN : C : O + NH_3$$

2. Hydrolytic stage:

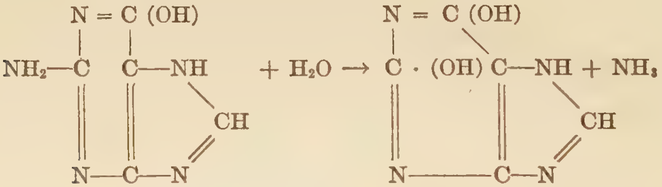
$$HN : C : O \longrightarrow NH_3 + CO_2$$

The function of the enzyme is to bring about the dissociation of urea into ammonia and cyanic acid; the hydrolysis of the latter follows as a secondary stage in the presence of water. The activity of urease is greatly stimulated by a co-enzyme which is thermostable,[648] and also by glycocoll. The synthetic action of urease has been described.[769]

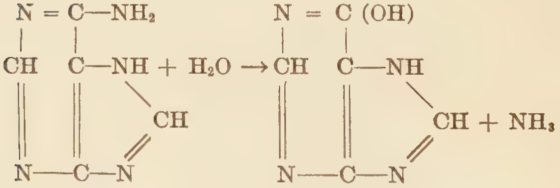
The *measurement of urease* is based upon its ability to liberate ammonia from urea. The enzyme preparation and some toluol are added to 50 or 100 cc. of a 1 per cent urea solution, and the mixture is incubated at 37°C. or at room temperature for a few days. As a control, the same amount of enzyme and toluol are added to 50 or 100 cc. of water. At the end of the incubation period, the ammonia is determined by adding Na_2CO_3 and aerating with a strong current into a standard solution of H_2SO_4 containing a proper indicator. The residual acid is titrated with standard alkali.[1321] The amount of salivary urease may be determined by a somewhat different method.[1095] To measure the action of gastric urease the following mixture has been used:[756] 5 cc. of 0.04 M urea, 3 cc. M phosphate mixture at pH 8.0, 1 cc. H_2O and 1 cc. of enzyme solution. This is incubated at 38°C. and the amount of ammonia, which is liberated, is determined after various intervals.

The optimal temperature for bacterial urease is 50°C. but even at this temperature, the enzyme is destroyed within a few hours; for soy bean urease, 55°C. is the optimum.[1213] The optimal reaction for the action of urease is shifted more and more to the acid side, with an increase in the concentration of urea; namely, from pH 7.9 to pH 7.2; this change is independent of the buffer concentration.[754] Sodium fluoride reduces the activity of urease;[1263] formaldehyde and other aldehydes are also injurious, while cyanides activate soy bean but not bacterial urease[607] by removing the injurious action of heavy metals.

v. *Purine amidases.* These enymes convert adenine to hypoxanthine (*adenase*) and guanine to xanthine (*guanase*). The *purine oxidases,* which oxidize hypoxanthine to xanthine, xanthine to uric acid, and uric acid to allantoin are allied to these enzymes. The purine amidases are well distributed in the animal organs and play an important rôle in the decomposition of nucleic acids. Their action is similar to that of nuclease:



Guanine + (*Guanase*) → Xanthine



Adenine + (*Adenase*) → Hypoxanthine

Both adenase and guanase are present in the liver and spleen of cattle and several other animals as well as in various human organs but in the pig spleen only adenase is present.[629,630,1086]

An aqueous extract of the macerated organ is usually sufficient as a source of enzyme. The fresh organ is finely macerated, pressed through a sieve, then ground with quartz sand or infusorial earth. Two to three parts of water are then added to one part of ground organ and the mixture is allowed to stand a few hours, in the presence of chloroform. The liquid is filtered and preserved, in the presence of toluol and chloroform. To purify the enzyme, sufficient ammonium sulfate solution should be added to make a 25 per cent concentration, together with enough sodium carbonate solution to keep the liquid slightly alkaline. After twenty-four hours, the liquid is filtered off and a sufficient amount of saturated ammonium sulfate added to make a $33\frac{1}{3}$ per cent concentration. After another twenty-four hours, the liquid is again filtered off. The liquid is then brought to 66.6 per cent saturation with $(NH_4)_2SO_4$. The precipitate is filtered off after twenty-four hours. This precipitate, which contains the enzyme, is suspended in 500 to 800 cc. of H_2O and shaken, in the presence of chloroform, for one-half to one hour. The liquid is then dialyzed until free from ammonia. This enzyme solution, which is almost free from purine bases, can be preserved.[1086]

To test the activity of the enzyme, 0.5 gram of guanine or adenine, dissolved in N NaOH, or 0.5 gram of adenine sulfate are mixed with 500 cc. of enzyme extract. Chloroform and toluol are added and the mixture incubated at 37°C. for five to ten days. The addition of toluol is of great importance, since it prevents the admission of air, which would lead to the further oxidation of the xanthine and hypoxanthine formed. At the end of the period of incubation, the amount of xanthine and hypoxanthine are determined by first freeing the solution from proteins by precipitation with acetic acid and then precipitating the purine bases by the copper bisulfate method.

vi. *Creatase and creatinase.* These enzymes decompose creatin and creatinin and are present in muscle, liver, brain, spleen, lung and certain other tissues. Creatin is formed during the autolysis of muscles and other organs; this, as well as any creatin added, is probably transformed by enzyme action into creatinin.[486] However, the existence of a specific enzyme which has this dehydrating action is still unproven.[805] The equilibrium between creatin and creatinin possibly depends upon the reaction of the muscle or organ.[506, 513]

Creatase may be extracted from macerated organs by the Buchner pressure method, or fresh organs can be macerated or finely divided, thoroughly ground with quartz sand and extracted for thirty minutes with physiological salt solution. If a longer period of extraction is used, toluol should be added, or the preparation placed in an ice chest. The sand and organ residue are then centrifuged off.

To determine the activity of the enzyme, 50 to 100 mgm. of creatin or creatinin are added to 25 to 50 grams of organ extract. Toluol is added and the mixture incubated, at 37°C. for three to six days. At the end of the period of incubation, the liquid mixture is poured into 150 to 300 cc. of a boiling 5 per cent NaCl solution. Acetic acid is then added until the reaction is distinctly acid and mixture boiled rapidly. The protein may also be removed by coagulating with alcohol.[805] The precipitated protein is filtered off and washed with boiling water. The combined filtrate is brought to the original volume and the amounts of creatin and creatinin determined.

d. Autolytic enzymes. The autolytic enzymes are probably a complex mixture of enzymes of particular groups of cells or tissues, which act upon the constituents of these cells and tissues, when placed under conditions which are favorable for their action. They have been discussed in Chapter V on tissue enzymes (see p. 86). The nature of the action of these enzymes depends upon environmental conditions, such as reaction, etc. Among the autolytic enzymes are those acting upon nucleins and their derivatives, and proteins and their degradation products, such as arginine, etc. The indefiniteness of their substrates is probably the reason that no specific autolytic enzymes are known in a pure state; they usually consist of infusions and juices of tissues, organs and cells. In the case of yeast, for example, autolysis represents a gradual decomposition of proteins, the optimal reaction being pH 6.1, lying between that of yeast pepsin, on the one hand (opt. = pH 4.0 to 4.5), and yeast trypsin (opt. = pH 7.0) and erepsin (opt. = pH 7.8), on the other.[315] According to one report,[180] organs contain a single autolytic enzyme whose optimum varies from pH 3 to 7. The organ or cell enzymes are often more or less specific, acting more or less selectively upon the constituents of the organs or cells from which they were derived.[603]

The transformation of protein nitrogen into amino acids and ammonia nitrogen is usually used as the index of the action of the autolytic enzymes and various methods are based upon this process.[181,182] This increase in amino nitrogen can be titrated.[202,451,1157,1212] Further information on autolytic enzymes and on the influence of organic and inorganic substances on tissue enzymes[1039] is obtainable elsewhere.[318,341,736,935,1138]

3. Proteolytic Enzymes of Plants. Proteolytic enzymes are widely distributed in the plant kingdom (see pp. 96 to 97), including seeds of various plants (certain Gramineae and Leguminosae), seedlings and various fruits.[444] They are capable of hydrolyzing

their own proteins and those of other plants, as well as certain animal proteins and various artificially prepared peptides; in other words, they are a mixture of proteases and peptidases. Some plant enzymes are distinctly of the pepsin type, acting in an acid medium and only upon proteins and not upon polypeptides;[3] this is especially true of insectivorous plants, such as certain species of Nepenthes[1266] and Utricularia.[20] Other enzymes, as for example those of germinated seed, are more tryptic in nature, both in reaction and in their hydrolytic products.[1227] However, the proteolytic enzymes of plant origin have not been as clearly differentiated as those of animal origin and it is possible that true tryptic enzymes are entirely lacking in the plant organs and various germinated seeds and that there is only a mixture of pepsin-like enzymes, or peptases, and erepsin-like enzymes, or ereptases. The optimal reaction for plant proteases is usually about pH 5.0, which coincides with the isoelectric point of protein, so the suggestion has been made that these enzymes attack undissociated protein and therefore differ from pepsin and trypsin which act upon the dissociated part of the protein.[1286]

a. Proteases of seed and other plant tissues. The proteolytic enzymes of seeds especially those of germinated seed (malt, etc.) have been extensively studied.[288, 496] An active enzyme can be obtained by grinding rolled oats for 10 to 12 hours in a ball mill with a mixture of equal parts of water and glycerin, then pressing out the liquid and allowing the sediment to settle. The brownish yellow liquid is removed, filtered and preserved on ice.[498] Other methods for the preparation of plant proteases have been described on pages 96 to 97. The optimal reaction for malt protease is pH 3.7 to 4.2.[761]

Measurement. The activity of seed proteases should usually be tested at several reactions; for example, three 100 gram portions of barley, oats, bean or vetch seeds are incubated from six to twenty-four hours at 37°C., with 1000 cc. of 0.2 per cent HCl, water, and 0.2 per cent Na_2CO_3 solution respectively. At the end of the period of digestion, the liquid in each of the three flasks is separated from the solid portion and made up to 1500 cc. with water. The total nitrogen is determined in 25 cc. The protein content of another 50 cc. is determined by coagulation with acetic acid. The remainder of the liquid is used for the determination of the various substances resulting from the decomposition of proteins, such as albumoses and nitrogen. One hundred grams of the material treated with cold water for ten minutes, should be analyzed as a control. The following is a typical result: 100 gms. of *Vicia faba* contains 4.4 grams total nitrogen, of which 17.4 per cent is soluble before incubation; as a result of digestion, at an alkaline reaction, 22.6 per cent is soluble, at a neutral reaction, 29.8 per cent and, at an acid reaction, 36.4 per cent. The activity of seed protease may also be determined as follows:[47] 30 grams of oats are treated with 300 cc. of water. One portion is boiled immediately and incubated, to serve as a control. The other is first incubated and then boiled. The increase in the soluble nitrogen serves as an index of the action of the enzyme. The activity of plant enzymes can be best studied by the increase in the amino nitrogen content of the enzyme-substrate mixture which can be determined by one of several methods.[202, 451, 1157, 1212]

b. Papain (papayotin) and related enzymes. Papain is an enzyme, present in the leaves, fruit, milk, juice, roots, trunk and seeds of *Carica papaya* and other species of Carica, which acts upon proteins.[958, 1309] The active enzyme can be obtained by pricking the fruits or extracting them in water, or through an incision in the trunk. The juice hardens in the presence of air. This hard product is ground to a powder and dissolved in water. The liquid is filtered and the filtrate precipitated with ten volumes of alcohol. The resulting precipitate is redissolved in water, reprecipitated with alcohol and then dried in vacuo. The enzyme can be purified by adsorption with aluminium hydroxide from an aqueous-alcoholic solution containing ammonia; it is then extracted from the precipitate by means of dilute acetic acid.[1286] Papain can be heated for thirty minutes at 70°C. without a perceptible diminution in enzymatic activity.[363] It is destroyed at 82° to 83°C. while pepsin is destroyed when heated for half an hour at 68°C. and trypsin at 60° to 65°C. The optimal temperature for the action of papain is very high (65°C. to 70°C.) and near the temperature of destruction.[631, 810, 984, 1070] The optimal reaction is pH 5.0.[458] The activity of papain is greatly increased by the addition of HCN and H_2S;[810] when activated with HCN, the enzyme is specific for certain substrates and differs from the inactivated enzyme; although papain is a true protease, it becomes capable of attacking peptone (peptidase) when activated by HCN. The maximal activation is obtained by allowing the cyanide to act upon the enzyme for one hour, then mixing it with the gelatin solution. The process of activation consists in the formation of a dissociable compound between the enzyme and the cyanide.[1286]

Bromelin, or the enzyme of pineapple, is prepared by cutting and pressing the fruit.[247] To each volume of pressed juice, two volumes of alcohol are added; the resulting precipitate is filtered off and discarded as it contains only traces of enzyme. To the filtrate five volumes of alcohol are added. The resulting coagulum is collected, dried and powdered. Bromelin may possibly consist of two enzymes, one resembling pepsin, which is destroyed at 65°C., and the other similar to trypsin but possessing greater resistance to toxic agents.[227] The optimal temperature for bromelin is high, 60°C. At 100°C. the enzyme is rapidly destroyed.[984]

4. HEMOLYSINS. The liberation of hemoglobin from the cell stroma, which results from the action of amboceptor and complement, is influenced by temperature in much the same manner as are enzymatic reactions, so that it is possible that hemolysis, in some instances at least, may be an enzymatic process. The optimal temperature for amboceptor-complement hemolysis is between 40° and 45°C.[487] The hemolysins and bacteriolysins of bacteria were described elsewhere (see pp. 109 and 113).

CHAPTER XII

OXIDASES AND OXIDO-REDUCTASES

1. OXIDATION SYSTEMS. The heterogeneous group of enzymes which bring about the processes of oxidation, reduction, the formation of organic acids, alcohols and carbon dioxide, and which take an active part in cell respiration and in the various fermentations and the activities that lead to a liberation of energy have been classified as *desmolases.*[888a] The hydrolytic enzymes prepare the substrate, while the desmolytic enzymes stimulate the processes by which the cells change the chemical energy of the nutrients, or of the cell constituents, to other forms of energy, especially heat and mechanical energy. However, some of the catalyzed processes belonging to this group do not yield any free energy, as in the action of carboxylase. The oxidative enzymes are commonly classified in the following five groups:

a. Direct oxidases, true oxidases or aero-oxidases, the enzymes which oxidize by means of free oxygen.

b. Indirect oxidases or hydroxidases—the oxido-reductases, the enzymes which can utilize oxygen in the form of water, bringing about oxidation and reduction at the same time.

c. Peroxidases—enzymes which cannot use molecular oxygen, but which act in the presence of hydrogen peroxide or certain organic peroxides.

d. Reductases or perhydridases (Bach[66]).

e. Enzymes of fermentation—the zymases and glycolytic enzymes.

However, this classification is not fully accepted at present, since' as a result of the most recent investigations, the distinction between direct and indirect oxidation is disappearing. Furthermore it is possible that the hydrogen rather than the oxygen, is activated whether free oxygen or a peroxide acts as an acceptor or an aldehyde group is changed to an alcohol.[1270a] Since the reaction of reduction takes place in coupled processes, according to the Cannizzaro reaction ($2R\cdot CHO + H_2O = R\cdot CH_2OH + R\cdot COOH$), the conception of an independent group of reductases is also being discarded. A detailed review of the older ideas on oxidation may be obtained elsewhere.[382]

According to Bach,[66] during slow oxidation, the oxygen molecule O = O is partly broken by the free energy of the utilizable substances to —O—O—, leading to the formation of peroxides:

$$H-O—O-H \text{ or } H_2 = O = O$$

Half of the oxygen in the peroxide is in a loosely bound active condition and is readily given off to other substances. The peroxides possess a much higher oxidizing potential than molecular oxygen and can serve to oxidize the excess of the auto-oxidizable substance (A) or some foreign substance (acceptor, B):

$$\begin{aligned} H_2 + O_2 &= H_2O_2 \\ H_2O_2 + H_2 &= 2H_2O \end{aligned}$$

In other words:

$$\begin{aligned} A + O_2 &= AO_2 \\ AO_2 + A &= 2AO \end{aligned}$$

or

$$\begin{aligned} AO_2 + B &= AO + BO \\ AO + B &= A + BO \end{aligned}$$

By means of the auto-oxidizable substances, the oxidation potential of oxygen in respect to different substances is increased.

Certain substances hasten the oxidation of auto-oxidizable substances by peroxides; they have been termed peroxidases and are sometimes considered to be of enzymatic nature. Nearly all animal tissues, leucocytes, milk, hemoglobin as well as certain metallic compounds, such as iron, copper, and manganese oxides, possess the function of peroxidases:

$$3H_2O_2 + Fe_2O_2 = 3H_2O + Fe_2O_5$$

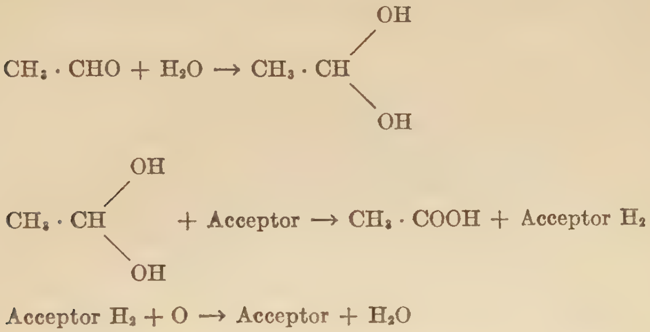
Fe_2O_5 gives off two atoms of oxygen and changes into Fe_2O_3; in so doing, a more active oxidation is accomplished than with H_2O_2 alone. If the substrate, or the auto-oxydizable substance is a chromogen, pigment formation takes place in the presence of a peroxide and a peroxidase.

An oxidation system, therefore, consists of two components: (1) oxygenase, or organic substance capable of forming peroxides, and (2) peroxidase, which increases the oxidizing potential of the peroxide and transfers oxygen from the peroxide to the substance to be oxidized. In addition to this system, there is catalase, which destroys the peroxide so that oxygen is given off. Both peroxidase and catalase act upon hydrogen peroxide and various organic peroxides, the former liberating oxygen in an active state, while the product of the latter is inactive oxygen, probably in a molecular state. Peroxidase alone in the absence of a peroxide is unable to bring about oxidation, but the presence of a peroxidase and peroxide, the system acting as an "oxidase," results in oxidation. The rôle of the oxygenase, an organic substance present in the cells, consists in activating molecular oxygen, producing organic peroxides. The enzyme part of the system is the peroxidase, which increases the oxidizing potential of the oxygenase; it is possible that oxygenase alone may oxidize various substances.

Potato juice can produce the guaiacum reaction (see p. 234), because it contains oxygenase and peroxidase; cucumber juice, however, which contains peroxidase alone, produces the guaiacum reaction only in the presence of H_2O_2.[1264] The peroxidase is often regarded as a colloidal hydroxide of iron or manganese, protected from precipitation by a stable hydrophile colloid, which decomposes the peroxide, liberating "active" oxygen, the latter possessing strong oxidizing properties. The active state of the nascent substance has been described as "the mode in which the energy of a reaction is enabled to be used for other chemical work before it has been degraded into heat."[108] Oxidizing processes have not been definitely proven to be enzymatic in nature; for example, the laccase of *Medicago sativa*, instead of being an oxidase, is sometimes referred to as a mixture of salts of organic acids and alkali earths;[407a] glycolic ($CH_2OH \cdot COOH$) and glyoxalic acids ($COOH \cdot CO \cdot COOH$) predominate among the former.

Iron probably plays a very important rôle in oxidation processes; molecular oxygen reacts with ferrous iron, producing a higher degree of oxidation.[1249a] This oxidized compound reacts with organic substances as a substrate giving back the ferrous iron. Molecular oxygen itself never reacts directly with organic matter, which is not auto-oxidizable, while iron is. The addition of 0.0001 to 0.00001 N HCN, due to the formation of an iron-HCN complex, will check oxidation in all processes in which oxygen is combined and transferred, such as in the assimilation of carbon dioxide or nitrates, in the decomposition of hydrogen peroxide or in respiration.

The process of oxidation may possibly consist not in the activation of oxygen, but of hydrogen; according to this hypothesis, oxidation consists in the taking away of hydrogen, while reduction consists in the addition of hydrogen:[1270]



$$CH_3\cdot CHO + H_2O \rightarrow CH_3\cdot CH\begin{matrix}\diagup OH\\ \diagdown OH\end{matrix}$$

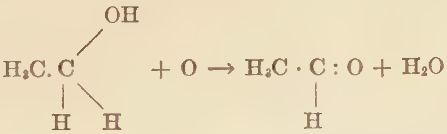
$$CH_3\cdot CH\begin{matrix}\diagup OH\\ \diagdown OH\end{matrix} + \text{Acceptor} \rightarrow CH_3\cdot COOH + \text{Acceptor } H_2$$

$$\text{Acceptor } H_2 + O \rightarrow \text{Acceptor} + H_2O$$

The reaction is the same even in the absence of free oxygen, if there is sufficient acceptor, such as methylene blue,[256a] to combine with the hydrogen liberated. For example, acetic acid bacteria will oxidize alcohol in the presence of methylene blue and in the absence of oxygen:

$$CH_3CH_2OH = CH_3\cdot CHO + 2H$$

This activation of hydrogen is shown in a general way in the process of oxidation of an alcohol (with oxygen as the hydrogen acceptor):



$$H_3C.\overset{\diagup OH}{\underset{|\ \diagdown\atop H\quad H}{C}} + O \rightarrow H_3C\cdot\underset{\underset{H}{|}}{C}:O + H_2O$$

The oxidation process thus becomes an hydration phenomenon and the rôle of oxygen consists in binding activated hydrogen; the place of oxygen can then be taken by other substances which have an affinity for hydrogen greater than that of the substance which is formed as a result of the withdrawal of hydrogen. Methylene blue thus acts as a hydrogen acceptor, in the oxidation of alcohol to acetic acid in the absence of oxygen, as well as in other reactions. The rôle of peroxidase may consist in the activation of hydrogen and in carrying it over upon the oxygen of the peroxide.[1270] This theory helps to explain the phenomena of oxidation, brought about not by the addition of oxygen, but by the reduction of hydrogen, as in the oxidation of succinic acid to fumaric acid by muscle enzymes, in the presence of methylene blue:

$$COOH \cdot CH_2 \cdot CH_2 \cdot COOH + M.\ B. = COOH \cdot CH{:}CH \cdot COOH + M \cdot B \cdot H_2$$

Oxidation and reduction thus become two conceptions of the same process, as will be shown in detail later.

The production of peroxide in the potato tuber is intimately associated with the lecithin of the tuber.[471] Various terpenes, which are among the unsaturated compounds present in plants, can combine with oxygen to form peroxides. The amount of enzyme in a plant depends upon the stage of its development, being at a minimum in the embryo, increasing with the development of the plant and then diminishing with the further growth of the organism.[942] Juices having oxidizing properties contain only a peroxidase, which acts only in the presence of peroxides. Any substance containing a peroxide linkage will activate a peroxidase, just as an acid or alkali, which increases the H^+ or OH^- concentration, will activate an hydrolytic enzyme. A plant heated to 60°C. does not produce the guaiacum reaction, because the peroxide is destroyed, but as the peroxidase is not injured, the oxidizing activity is restored if a peroxide is added.

Various oxidases are specific in nature, for example, laccase acts upon hydroquinone or pyrogallol, but not upon resorcinol or tyrosine. The latter substance is acted upon by *tyrosinase*.

2. OXIDATION, RESPIRATION AND FERMENTATION. Respiration is usually distinguished from fermentation by the fact that in the former, energy is utilized by oxidation processes, while in the latter, energy is utilized by the decomposition of complex compounds into those which are simpler. In complete oxidation, all of the potential energy is completely changed into kinetic energy:

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O\ (+\ 674\ \text{Calories})$$

In fermentation, compounds are formed which still contain a supply of potential energy:

$$C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2\ (+\ 27\ \text{Calories})$$

The respiration process in plants may be carried out according to the following reactions, in which *R* represents the respiratory chromogens or hydrogen acceptors which are capable of absorbing activated hydrogen:[942]

$$C_6H_{12}O_6 + 6H_2O + 12\ R = 6CO_2 + 12\ RH_2$$
$$12\ RH_2 + 6O_2 = 12H_2O + 12\ R$$

or

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O$$

In the process of respiration, carbohydrates are decomposed anaerobically into carbon dioxide and incompletely oxidized organic compounds; half of the oxygen is obtained from the glucose and half from the decomposition of water, which plays a chemically active part in respiration. The oxidases have no rôle in the first phase of respiration but the reductases are active, and as a result of their action the hydrogen of the glucose is activated. The hydrogen is thus either used up in reducing some incompletely oxidized compounds or it is oxidized aerobically, with the formation of water. All of the oxygen absorbed in respiration is used exclusively for the oxidation of the hydrogen, extracted by the hydrogen acceptors, and is separated out as water. The acceptors are thus regenerated. The oxygen itself becomes the final hydrogen acceptor. However, the oxidation of the hydrogen acceptors by the atmospheric oxygen cannot always take place by itself as in case of methylene blue (the analogy between methylene blue and oxidase is described elsewhere[54]); in some cases, an oxygen activator is required, which is an oxidase. The rôle of oxidizing enzymes is believed to consist in this function of activating the oxygen for the oxidation of the hydrogen acceptors. These reactions can be represented schematically:

$$C_6H_{12}O_6 \rightarrow \text{products of primary decomposition} \rightarrow \text{(reductase)} \rightarrow$$
$$2H \rightarrow 2H + R \rightarrow RH_2 \rightarrow RH_2O \rightarrow R + H_2O$$

The following substances can act as donators in plant respiration: Sugars and sugar derivatives, including aldehydes, acids, alcohols, methyl glyoxal, amino acids, purines, and phenol. Among the acceptors, are various tissue substances, including aldehydes, methylene blue, thio-groups ($R \cdot SH$), nitrate and aromatic nitro-bodies.

Cell respiration may also be carried on by a thermolabile enzyme and a thermostable co-enzyme.[819] The latter is readily soluble in water, while the enzyme itself, due to its colloidal nature, is closely connected with the protoplasm of the cells. The enzyme and co-enzyme can be separated by washing and centrifuging or by filtration through a gelatin filter. Various narcotics and cyanide greatly diminish the process of respiration. Muscle tissues and acetone-treated-yeast both act as co-enzymes and are physiologically equivalent. The process of the transformation of carbohydrates in animal respiration and in alcoholic fermentation proceeds similarly through the stage of hexose-phosphoric acid; but in the muscles, lactic acid is an intermediate product, and in case of yeasts, CO_2 and alcohol. In muscle respiration, the oxidases activate the oxygen, and CO_2 and H_2O are the final products. In alcoholic fermentation, which is a purely anaerobic process, reductases activate the hydrogen.

The fundamental difference between cell respiration and fermentation thus disappears; they are not two distinct processes but merely two steps of the same process. Whether only the first stage (fermentation) takes place or whether the second also follows depends on conditions. Alcoholic fermentation of yeasts, lactic acid fermentation of bacteria, glycolysis of animal tissues and other fermentations, as well as partial or complete oxidation of all organic substances in metabolism, are only phases of the same group of fermentative reactions. In some cases much more of the intermediary products are formed in the process of fermentation than can be oxidized. A part of these products are then synthesized again to the original substrate.[820a]

3. Oxidase and Peroxidase. The catalytic nature of oxidizing and reducing enzymes is still a matter of discussion. With the advance of the knowledge concerning these phenonena, it has become evident that some of the processes of oxidation which occur in the cells are not the result of the action of catalysts; substances like oxygenase and peroxidase, only recently considered as oxidizing enzymes, begin to lose the attributes of enzymes.

Preparation. Oxidase is usually extracted from plant tissues, by grinding and extracting them, for one to two days, at room temperature, with chloroform water and then filtering. This enzyme can be concentrated by precipitation with 3 volumes of 95 per cent alcohol. The precipitate is filtered off, washed with alcohol and dried in vacuo at 40°C. An oxidase may also be prepared by grinding up freshly washed potatoes and adding 0.5 to 1 per cent acetic acid.[1149] After twenty-four hours, the extract is filtered and the clear filtrate saturated with ammonium sulfate. The resulting precipitate is filtered off, washed with cold saturated ammonium sulfate solution and then dissolved in water. This precipitation and solution are repeated three or four times. The solution is finally dialyzed in a parchment bag in flowing water and then precipitated with 4 to 5 volumes of 95 per cent alcohol. This precipitate which contains the oxidase is washed with ether and dried over sulfuric acid in a desiccator. A purified enzyme may be obtained by dissolving the last precipitate in water and reprecipitating with alcohol.

Oxidase may be obtained from fresh vegetables by scraping the skin in the case of carrots and turnips, pealing in case of potatoes, or removing the outer leaves in case of cabbage.[422] The vegetables are then weighed, ground in a meat chopper and squeezed through muslin by hand. If dehydrated vegetables are employed, they are first soaked for 20 minutes in 13 times their weight of water, then chopped, ground, allowed to extract for 30 minutes, and then squeezed. The pressed juice can be used as the source of oxidase or it can be purified by precipitation with five volumes of alcohol. The resulting precipitate may be redissolved in water and reprecipitated.

Peroxidase is prepared by grating potato tubers into a pulp, mixing with calcium carbonate to neutralize the acids, then grinding with quartz sand in a mortar for two minutes and filtering through muslin.[37] The oxidase is removed by raising the temperature to 70°C. for ten minutes; the peroxidase is not injured. The oxidase may also be destroyed by the addition of acetone. The amount of peroxidase in potato juice is relatively greater than that of oxidase, therefore, if a high dilution is used, the amount of the oxidase present is negligible.

A purified peroxidase can be obtained by washing 5 kgm. of finely sliced horse radish for a few days in running water to remove soluble substances by dialysis.[1275] The material is then warmed with a 0.4 per cent solution of oxalic acid for a few hours to precipitate the enzyme. The residue is then crushed, washed on a filter paper with 15 liters of very dilute oxalic acid solution, and then ground with baryta water to liberate the enzyme. The liquid is pressed out and treated with carbon dioxide to precipitate the barium. Upon the addition of alcohol, a slimy substance is precipitated out. The filtrate is concentrated to 50 cc. by evaporation and the enzyme precipitated by the addition of five volumes of alcohol. The enzyme is then purified by redissolving in water containing a trace of sulfuric acid and reprecipitating with alcohol. The product is a mixture of the enzyme and a nitrogenous glucoside; the latter can be precipitated with mercuric chloride and the filtrate again precipitated with alcohol. This process should be repeated several times. The final yield of the enzyme is 0.45 gram, or 60 per cent of the theoretical total amount. The optimal reaction for the peroxidase of horse radish is pH 4.5 to 4.75.

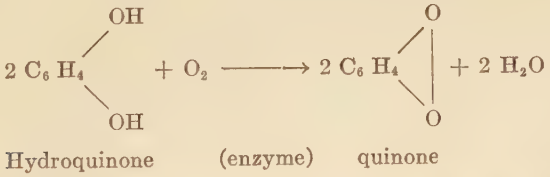
Distribution. Oxidases and peroxidases are widely distributed in the animal, plant and microbial kingdoms.

Measurement. Both oxidase and peroxidase act upon the same substances, but the presence of H_2O_2 is always required in the case of peroxidase. A large number of substances can be used for measuring the action of these enzymes, among which are, tincture of guaiacum, aloin, α-naphthol, benzidin, malachite green, α-and p-amidophenol, hydroquinone, pyrogallol, resorcin and formaldehyde. The guaiacum reaction is performed by treating a freshly prepared tincture of guaiacum or guaiaconic acid solution (0.3 gram in 10 cc. of alcohol), in the presence and in the absence of hydrogen peroxide (2 cc. of a 3 per cent solution), with 1 cc. of the enzyme solution. A blue color formed, in the absence of H_2O_2, indicates oxidase. If the blue color is formed only in the presence of H_2O_2, peroxidase alone is present. For quantitative results, the color can be matched with a standard blue color of a solution of indigo carmin.

The "purpurogallin number" or the number of milligrams of purpurogallin which are produced from pyrogallol and H_2O_2 by 1 mgm. of the vacuum dried preparation has been suggested as an index of enzyme action.[1275] This number is 0.25 for well ground horse-radish, 360 for the crude enzyme (before purification with $HgCl_2$) and about 670 for the purest enzyme preparation. The method is a follows: a 1 per cent aqueous solution of p-phenylenediamine HCl and a 1 per cent solution of α-naphthol in 50 per cent alcohol are kept separately and mixed in equal volumes before using. Five drops of this reagent are added to a few cubic centimeters of the enzyme solution and the color formed is observed after one-half and fifteen minutes. It is pink at first, then a deeper shade and finally deep red purple. The quinone formed in the oxidation of guaiacol by oxidase can be extracted in chloroform and its amount determined by comparison in a colorimeter with a N/10 iodine solution; a 10 mm. layer of this solution corresponds to 46.5 mgm. of guaiacoquinone per liter.[448]

The oxidation of a weakly acid solution of KI by H_2O_2 is greatly hastened by the addition of peroxidase. The amount of iodine formed can then be determined by titrating with N/100 thiosulfate solution, using starch as an indicator.[61a] The procedure is as follows: 5 cc. 2 per cent KI solution, 1 cc. 1 per cent acetic acid and 0.5 cc. 1 per cent starch solution are placed in each of two flasks. The flasks are then placed in a bath at 20°C. and 2 cc., or a larger amount, of the enzyme solution are placed in one flask and a corresponding amount of distilled water in the other. One cubic centimeter of a dilute H_2O_2 solution (5 drops of a 3 per cent solution in 20 cc. distilled water) is then added to each flask. After 10 to 30 minutes, both flasks are titrated with N/100 $Na_2S_2O_3$ solution, until the blue color has disappeared. By substracting the control, a measure of the action of the enzyme is obtained. When oxidases are tested, no H_2O_2 is added. The action of oxidase and catalase may also be measured in a special apparatus.[532]

4. Phenolase (Laccase). Enzymes which are capable of oxidizing aromatic amines and phenols, with the formation of a pigment, belong to this group. They are also peroxidases in character and their action depends upon the presence of a peroxide. The latter serves as a consumer of the hydrogen which is activated in the phenols:[1270]

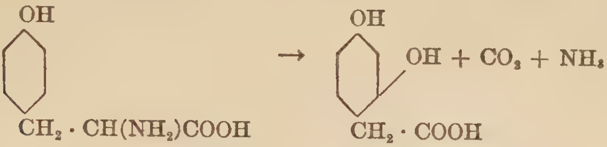


Phenolase occurs in certain animal secretions, such as saliva, nasal secretions and tears; in leucocytes; in worms, mussels, and other lower organisms; in various higher plants and is especially abundant in several of the higher fungi (*Russula*, etc.). Laccase can be isolated from the juice of the lac tree (*Rhus vernicifera*) by precipitating it with 6 to 8 volumes of alcohol, filtering and washing the precipitate with alcohol.[128a] The residue is then redissolved in water, reprecipitated with an excess of alcohol, washed and dried in vacuo. A white substance soluble in water and glycerin and rich in laccase is thus obtained. The enzyme can also be obtained by precipitating the juice with acetone.[448] Laccase when obtained from higher plants and fungi is usually associated with tyrosinase. The former acts best at a weakly acid reaction and the latter at a weakly alkaline one. They can be separated from one another by the following method:[128a] *Russula delica* is extracted at room temperature with chloroform water and the filtered extract then precipitated with 1½ volumes of alcohol. The precipitate which contains the tyrosinase can be dissolved in water, reprecipitated with alcohol and redissolved. The alcoholic filtrate contains the laccase, which can be concentrated at 50°C.

A phenolase can be obtained from *Lactarius vellereus*, by extracting the crushed fungus with water for three minutes at 70°C. while constantly stirring.[70] The tyrosinase is destroyed at this temperature but the extract is very rich in phenolase. One drop of this extract in 5 cc. of a 2 per cent guaiacol solution will cause the immediate appearance of a blue color, followed by the formation of a red brown precipitate, for in a mixture of α-naphthol and paraphenylenediamine, indophenol is immediately formed. The enzyme may be concentrated by the addition of sufficient magnesium sulfate to make a 2 per cent solution and then adding an equal volume of alcohol. The resulting precipitate is filtered off and three volumes of 98 per cent alcohol are added to the clear filtrate. The final precipitate is filtered off by suction, washed with 95 per cent alcohol and dried in vacuo over calcium chloride.

The quantitative *measurement* of the action of phenolase is based upon the colorimetric estimation of the greater amount of pigment formed in the presence of the enzyme than in the control;[70] guaiacol, pyrogallol or a mixture of α-naphthol and paraphenylenediamine (indophenol reaction[121]) and other aromatic compounds with two hydroxyl or amido groups, preferably in the *o*- and *p*-position, may be used to demonstrate this reaction. The activity of laccase can be measured by adding 10 mgm. of the purified preparation to 10 cc. of 1 per cent guaiacol and estimating the depth of the color produced.[448] At pH 6.7 and 30°C., if the solution is aerated, this amount of enzyme produces, in ten minutes, 4.5 to 5 mgm. of guaiacoquinone, which represents an absorption of 0.4 cc. of oxygen. The optimal reaction for laccase is pH 6.7, while the limiting reactions are pH 4.5 and pH 9.0.[448] Further information on the influence of acids upon phenolase[72] and on the formation of a specific antiphenolase may be obtained elsewhere.[69]

5. TYROSINASE. The action of tyrosinase upon tyrosine results in the formation of CO_2 and NH_3:



This reaction is divided into three stages: (1) the conversion of tyrosine into a red substance, for which oxygen and the enzyme are required, (2) the conversion of the red substance into a colorless substance (this change is spontaneous and occurs rapidly on warming; the presence of tyrosinase is not required) and (3) the oxidation of the colorless substance by oxygen into the dark colored substance, melanin.[1002] This last conversion may take place spontaneously but it is also accelerated by the phenolase present in the tyrosinase. Potato tyrosinase is active from pH 5.0 to 11.0.[251] At pH 7.0 or in acid solutions, the main product formed during the first six hours is the red substance, but, in alkaline solutions, the conversion of the red substance into melanin is very rapid.

Tyrosinase also oxidizes other substances chemically related to tyrosine (hydroxy-benzol derivatives), such as *p*-oxyphenylethylamine, *p*-oxyphenylamine, *p*-cresol, phenol and glycyl-tyrosine, but it does not affect phenylalanin or phenylethylamine.[129a] Tyrosinase acts more rapidly upon *l*-tyrosine than upon the *d*-form, the activity against the *dl* compound being midway between these two.[14] Further information on the tyrosinase-tyrosine reaction may be obtained elsewhere.[4,120,334,1002]

Occurrence. Tyrosinase is present in insects, worms, in the skin of various animals, in clover, potatoes and other plants. Several higher fungi, such as *Russula* and *Agaricus* and certain Actinomycetes (*Asp. scabies*) also form tyrosinase.[1111] The blood of the larvae of *Actia caja* contains an enzyme, which converts 3,4-dihydroxy-phenyl-alanin, in the presence of oxygen, into a black pigment.[1088]

Preparation. The juice of the animal or plant organ is extracted by means of a Buchner press, or with water containing toluol or chloroform. If tyrosinase is present, the addition of tyrosine will lead to the formation of a dark-brown pigment. Tyrosinase is separated from phenolase by the above method.

Measurement. To measure the action of tyrosinase, the methods of sedimentation and of spectroscopic analysis may be used.[469] The first is performed by centrifuging and measuring in a graduated tube with a fine point the volume of the precipitate; in the second, the pigment (melanin) formed as a result of the action of the tyrosinase is measured spectroscopically. The second method is much more accurate and can also be used for measuring small amounts of the enzyme. The amount of melanin formed may also be titrated with a permanganate solution;[63] 0.05 gram of tyrosine are dissolved in 100 cc. of a 0.04 per cent Na_2CO_3 solution by warming. Ten cubic centimeters of the enzyme solution, 10 cc. of the tyrosine solution and 30 cc. of water are mixed and incubated for twenty-four hours. One cubic centimeter of 10 per cent H_2SO_4 is then added and the solution titrated with 0.002 N permanganate solution until the dark melanin color disappears. A control is also made and substracted. The amount of permanganate required is a good index of the action of the enzyme. One-tenth per cent quinine sulfate solution may be used as a substrate for the action of tyrosinase, which results in the formation of a black color.[150] By immersing the plant containing the tyrosinase in the quinine solution, the cells are injured by the high concentration, and the enzyme is liberated.

6. Luciferase. The production of light by the mollusk *Pholas* is due to the oxidation of a light-producing substance *luciferin* by oxygen and water, in the presence of an enzyme *luciferase*.[250] The enzyme but not the substrate is destroyed on boiling. Luciferase is obtained by extracting the luminous animal with cold water; the luciferin is also extracted, but disappears on oxidation:

Luciferin + oxygen = oxyluciferin + water

The luciferin may be regenerated by reduction and can again be used as a substrate for the action of luciferase.[529] Luciferin is prepared by adding hot water to the luminous organ of the animal or by quickly heating the luminescent extract of the luminous animal to temperatures which permanently quench the light, or by boiling. The luciferase is thus destroyed before the luciferin (which is not destroyed by heating) has been completely oxidized. The luciferin-luciferase reaction was found in only 4 of the 16 groups of luminous organisms investigated (fireflies, *Pholas*, ostracods and *Odontosyllis*).[529] The luciferase of one animal may not be able to act upon the luciferin of another animal, unless these are closely related. The intensity of luminescence (or reaction velocity) is proportional to the concentration of luciferase and presumably also of luciferin, the reaction being monomolecular.[32]

7. Oxidation of Substances Consisting of Simple Carbon Chains. A number of processes of oxidation of simple carbon compounds are known, but the rôle of enzymes in the majority of these reactions is still an unknown factor. The following are examples of these processes: the transformation of sugar by fungi to citric and oxalic acid, the oxidation by fungi of various organic acids (lactic, tartaric, malic, mandelic and β-hydroxy-butyric),[558] the oxidation by bacteria of ethyl alcohol to acetic acid, of propyl alcohol to propionic acid, of sorbite to sorbose, of glycerin to dioxyacetone, erythrite to erythrose, and the oxidation by various animal tissues of alcohol, acetic, malic, succinic and other acids. The action of these oxidases is closely connected with metabolic processes, but very few of them can be demonstrated in enzymatic preparations.

Alcoholoxidase oxidizes ethyl alcohol to acetic acid and can be demonstrated in acetone-ether preparations of acetic acid bacteria:[209,210]

$$C_2H_5\cdot OH + O_2 = CH_3\cdot COOH + H_2O$$

Acetaldehyde (CH_3COH) is an intermediary product of this reaction. The optimal concentration of alcohol for this process is 4 per cent; calcium carbonate should be present to neutralize the free acids.

Animal tissues form an alcoholoxidase, which oxidizes ethyl, propyl, and methyl alcohols, glycol, benzylalcohol and saligenin, but not glycerin;[94] molecular oxygen is used. The optimal temperature is 55°C. at a weakly alkaline reaction, 40°C. at a greater alkalinity.

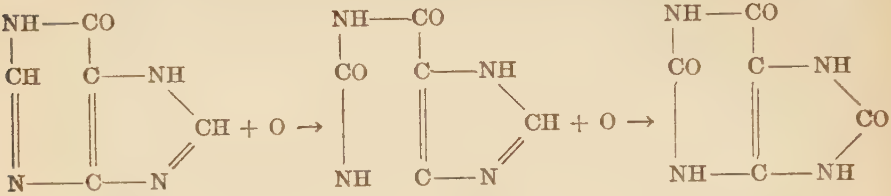
Direct oxidation of sugar has been observed with microörganisms, but not with animal tissues or fluids (the enzymatic nature of the oxidation of sugar to glycuronic acid is still unknown).

β-oxybutyrase, an enzyme oxidizing the ammonium salt of β-oxybutyric acid to aceto-acetic acid, has been demonstrated in aqueous extracts of the liver of dogs.[1225] It is thermolabile and can be precipitated with ammonium sulfate. The action of the enzyme is increased by the addition of blood or crystalline oxy-hemoglobin. The addition of acetic acid injures the enzyme action and small quantities of sodium carbonate stimulate it.

Acidoxidases are found in acetone-ether preparations of fungi;[558] they form carbon dioxide from *d*-tartaric acid, and to a lesser extent from *l*-tartaric acid. Compounds without an asymmetric carbon atom are not affected. Wheat flour, even after treatment with ether, can oxidize oxalic acid to carbon dioxide;[1316] fresh animal tissues can oxidize various organic acids.[94–96]

The various processes of tissue respiration, resulting in the formation of carbon dioxide, should also be mentioned. They are somewhat better understood in plants; a detailed review has been published.[287]

8. Purine Oxidases. These are found in various organs (spleen and lungs of cattle and the liver of horses and hogs). Their action is as follows:

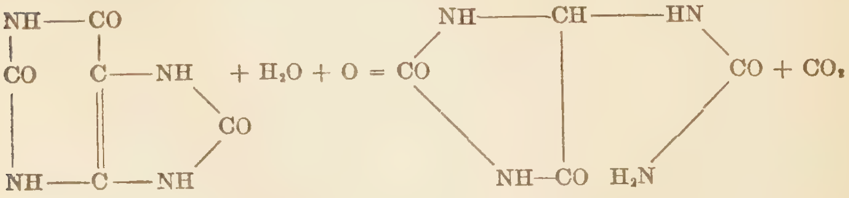


Hypoxanthine Xanthine Uric acid

Purine oxidase solutions can be prepared from cattle liver by extracting one part of ground liver with two parts of chloroform water at ice temperature for one to two days. The liquid is filtered and the filtrate can be used as the source of enzyme or it can be fractionally precipitated with ammonium sulfate, as described for the purine desamidases. This treatment produces an enzyme solution free from nucleoproteins and purine bases.

The action of the enzyme is determined by adding the extract to hypoxanthine or xanthine and aerating the mixture, so as to provide sufficient oxygen. After a certain period of incubation (ranging from a few hours for cattle spleen, to 3 or 5 days, for other material), the amount of uric acid formed is determined. Since most extracts contain both the desamidase and oxidase, guanine and adenine can be used in place of hypoxanthine or xanthine.[1086]

Uricase, the enzyme which is capable of oxidizing uric acid, in the presence of oxygen, to allantoin and carbon dioxide, belongs to this group of enzymes. Its action is as follows:



Uric acid Allantoin

An extract of uricase can be obtained from organs, especially from cattle brains by the methods previously described for the preparation of purine oxidases. The organ is thoroughly ground and shaken for fifteen minutes with 2.5 volumes of slightly alkaline water;[92] the liquid is then pressed through a cloth and centrifuged. This turbid liquid is diluted with 2.5 volumes of alcohol, and the resulting precipitate rapidly centrifuged, since the enzyme is very sensitive to alcohol, then washed with ether and dried in the air. The action of the enzyme is 2 to 3 times stronger if it is dried in pure oxygen instead of in air.

9. Aldehydase (Salicylase). The ability of plant and animal tissues to reduce dyes to leucobases, nitrates to nitrites, etc. is due to the combined action of an enzyme and a co-enzyme. The enzyme is known variously under the names of salicylase, aldehydase, oxido-reductase, aldehydecatalase, dehydrogenase, Schardinger enzyme, perhydridase and oxihydrase, and is found in milk, separate from its co-enzyme. The latter is extracted from tissues by boiling water and can be replaced by an aldehyde. The keto-compounds and the amino acids formed in the decomposition of proteins also give the aldehyde reaction. Aldehydase may be regarded as the connecting link between the oxidases and reductases. It oxidizes an aldehyde to an acid, for example, salicyladehyde to salicylic acid. However, no free oxygen is used in this reaction; the enzyme acts both as an oxidizing and reducing agent, transforming the aldehyde, on the one hand, to an acid and, on the other, to an alcohol:[94,951]

$$2\,R\cdot CHO + H_2O = R\cdot CH_2OH + R\cdot COOH \text{ (Cannizzaro reaction)}$$

Schardinger[1081] observed that although fresh milk alone had no action on methylene blue, the addition of formaldehyde led, at 70°C., to a reduction of the dye to its leucobase. Boiled milk was inactive. Further studies have shown that the reduction is due to an enzyme, known as the Schardinger enzyme, and that formaldehyde can be replaced by another aldehyde. The presence of this enzyme in milk is independent of bacterial activities;[1204a] it is found in normal milk and in colostrum and acts best at a weakly alkaline reaction. The reaction also takes place when, in place of methylene blue, a nitrate acts as the acceptor for hydrogen, the nitrate being reduced to nitrite.[64–67] The reaction may be considered as one of oxido-reduction, at the cost of the elements of water, in which the aldehyde is the oxygen acceptor and the pigment or nitrate is the hydrogen acceptor. Methylene blue can also be discolored in the presence of xanthine or hypoxanthine, which are oxidized to uric acid; the name perhydridase has been applied to the enzyme concerned in the Schardinger reaction.[64–67] It may be obtained by extracting calf's liver with a 2 per cent solution of sodium fluoride and a 1 per cent solution of sodium bicarbonate; the enzyme is then precipitated by means of alcohol. Perhydridase may also be prepared by precipitating 1 part of buttermilk with three parts of freshly distilled acetone; the precipitate contains the enzyme in a concentrated form.[1079]

To measure quantitatively the action of perhydridase, a solution of acetaldehyde and of sodium nitrate (free from nitrite) may be added to a definite amount of milk and the mixture heated in a closed vessel at 60°C. for thirty minutes.[64–67] A few cubic centimeters of a concentrated solution of basic lead acetate are then added, to precipitate the proteins, and the mixture filtered; the amount of nitrite in the filtrate is determined by titration with α-naphthylamine and sulfanilic acid (in acetic acid). The optimal temperature for the action of the enzyme is 65°C., the maximal amount of nitrite being formed in ten minutes. Further information on the Schardinger reaction may be obtained elsewhere.[187,325]

Aldehydases also occur in various animal organs, such as pancreas, liver, lungs, brains, spleen. The organs, especially the liver, of young animals contain more of this enzyme than those of old animals. It has not yet been demonstrated in plants. They can be prepared from fresh cattle liver by maceration with quartz sand; distilled water containing toluol is added and the mixture allowed to stand for a few hours, with frequent shaking.[601] The extract is then squeezed out and filtered. The dark clear liquid is treated with sufficient ammonium sulfate to produce 25 per cent saturation, and sodium carbonate is then added until the liquid is faintly alkaline. A slight precipitate is formed, settling in twenty-four hours; this is filtered off. Additional ammonium sulfate is added to produce a $33\frac{1}{3}$ per cent concentration and the resulting precipitate again filtered off after twenty-four hours. More ammonium sulfate is then added to the clear liquid to produce 60 per cent saturation. The heavy precipitate which settles in 24 hours contains the aldehydase. It is filtered off, washed with 60 per cent $(NH_4)_2SO_4$ and dissolved in distilled water. The solution is filtered, after a few hours, and treated with sufficient 95 per cent alcohol to produce a 30 per cent concentration. The precipitated enzyme is filtered off and extracted with diminishing quantities of distilled water containing a few drops of dilute sodium carbonate. The combined extracts may then be treated, at a weakly alkaline reaction, with a dilute solution of uranyl acetate until a precipitate is formed. This is extracted with water and a clear enzyme solution obtained.

Measurement. The action of salicylase may be demonstrated by the addition of ferric chloride to salicylaldehyde. The formation of a dark brown pigment indicates that salicylic acid has been produced.[1298] The optimal temperature is 60°C.; at 100°C. the enzyme is rapidly destroyed.

10. Reductases. These are differentiated from the enzyme concerned in the Schardinger reaction (perhydridase) by the fact that no oxygen-acceptor (aldehyde) is known; the tissues function as an acceptor of oxygen. These enzymes can be divided into three classes:[64] (1) those that act on water and are concerned with hydrolysis; (2) those that act on *oxygenated water* (H_2O_2) and are concerned with processes of oxidation and (3) those that act on hydrogenated water (H_4O or oxygen perhydride) and are concerned with processes of reduction. Just as the oxidases are compound systems of peroxidases and unstable organic peroxides, so are probably the reductases compound systems of perhydridases and unstable organic perhydrides. By giving up the excess of hydrogen, the perhydride activated by the perhydridase reduces various substances. Animal organs contain substances, which in themselves do not possess any reducing properties, but which can reduce dyes and nitrates, when perhydridase is present.[65] These co-enzymes are thermostable and can also be obtained from yeast preparations. There is a close correlation between the zymase and reductase content of yeasts.[765] Both consist of two parts—a thermolabile enzyme and a thermostable co-enzyme. Reduction takes place only when the two are combined. The addition of phosphates greatly stimulates the action of both enzymes.

The presence of reductases has been demonstrated in a number of microörganisms. The reduction of dyes, nitrates, sulfates and arseniates belongs to this group of reactions. Xanthine-oxidase, for example, will reduce nitrates to nitrites in the presence of hypoxanthine, xanthine, adenine or aldehyde; the nitrate takes the place of methylene blue.[328] The subject of purine oxidases has also been discussed on page 240, and further information may be obtained elsewhere.[848]

CHAPTER XIII

ZYMASES

Zymases comprise a group of enzymes which hydrolyze sugar, and are frequently separated into those which act upon the sugar itself (*hexases*) and those that bring about the consecutive steps in the transformation (*glyoxalase, carboxylase, et cetera*).

1. ZYMASE. The zymases are enzymes which convert glucose and a few other carbohydrates, through a group of chemical reactions, into carbon dioxide and water, if oxygen is present, or into alcohol and carbon dioxide or into lactic acid, if oxygen is absent. These enzymes are present not only among yeasts and bacteria, as was originally believed, but also in higher plants and probably in animals. Various theories have been proposed to explain the origin of alcohol: according to one hypothesis, alcohol passes through the lactic acid stage;[209] according to another, the sugar is first hydrolyzed into one molecule of glycerinaldehyde and one molecule of dioxyacetone, and the former passes, through the pyruvic acid and acetaldehyde stage, into ethyl alcohol.[714] The dioxyacetone combines with phosphate to form, first, hexose-phosphate and then hexose again:

$$\begin{aligned} 2C_3H_6O_3 + 2\,R_2HPO_4 &= 2C_3H_5O_2R_2PO_4 + H_2O \\ 2C_3H_5O_2\,R_2PO_4 &= C_6H_{10}O_4\,(R_2PO_4)_2 \\ C_6H_{10}O_4\,(R_2PO_4)_2 + H_2O &= C_6H_{12}O_6 + 2R_2HPO_4 \end{aligned}$$

The formation of hexose-phosphate and its decomposition is said to be a result of the action of two enzymes *phosphatese* and *phosphatase*.[406]

A third theory has been suggested in which the Cannizzaro reaction plays an important part, and trioses are not formed at all, the sugar changing directly into methyl-glyoxal:[882,886]

$$C_6\,H_{12}\,O_6 = \underset{\text{aldol-methyl-glyoxal}}{C_6\,H_8\,O_4} + 2\,H_2O$$

$$C_6\,H_8\,O_4 = \underset{\text{methyl-glyoxal}}{2\,CH_3 \cdot CO \cdot CHO}$$

$$2\,CH_3\cdot CO\cdot CHO + 2H_2O = \underset{\text{glycerin}}{CH_2OH\cdot CHOH\cdot CH_2OH} + \underset{\text{pyruvic acid}}{CH_3\cdot CO\cdot COOH}$$

$$CH_3\cdot CO\cdot COOH = \underset{\text{acetaldehyde}}{CH_3\cdot CHO} + CO_2$$

$$CH_3\cdot CO\cdot CHO + CH_3\cdot CHO + H_2O = CH_3\cdot CO\cdot COOH + CH_3\cdot CH_2OH$$

The acetic acid may arise from the acetaldehyde. The lactic acid is formed from the methyl-glyoxal:

$$\underset{\text{methyl-glyoxal}}{CH_3\cdot CO\cdot COH} + H_2O = \underset{\text{lactic acid}}{CH_3\cdot CHOH\cdot COOH}$$

This last process has been later found to be due to a specific enzyme, *methyl-glyoxalase.*[820] Further information on the chemistry of the formation of alcohol from sugar may be obtained elsewhere.[923]

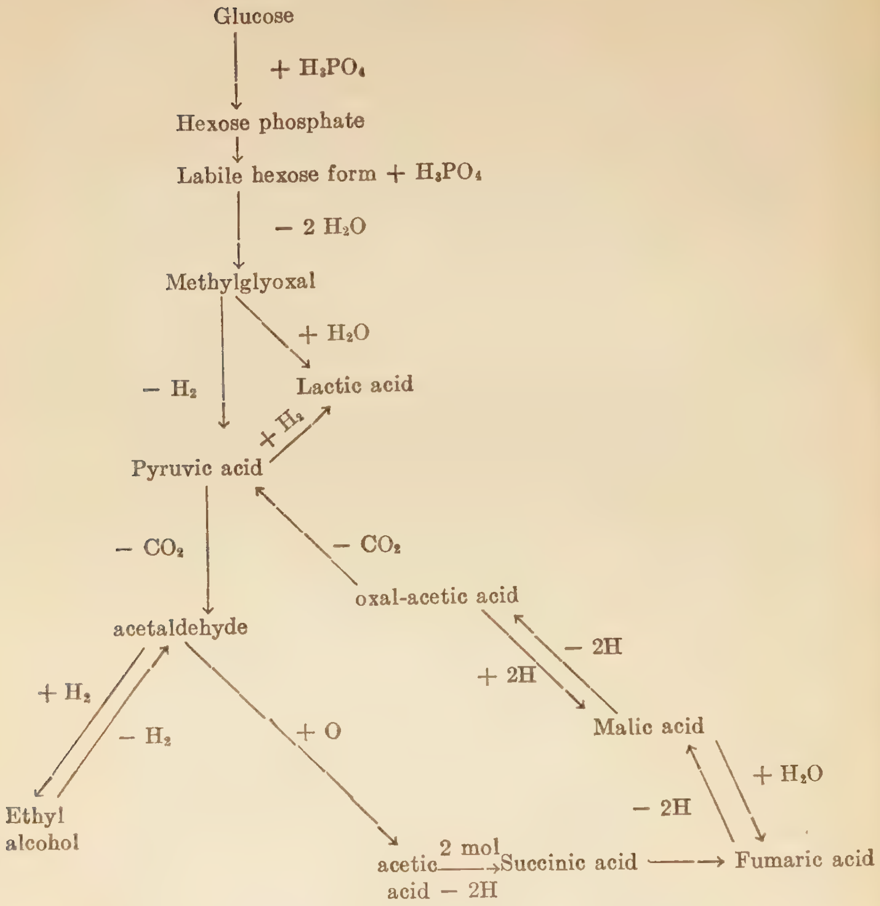
Phosphates play an important rôle in the fermentation process, as shown above.[519] It is possible that hexose-di-phosphate acts as a catalyst in the first stage of fermentation, the process being one of autocatalysis.[819] Arsenates greatly accelerate fermentation by stimulating the cleavage of the hexose-di-phosphate.[519] A series of reactions very similar to alcoholic fermentation has been described in the oxidation of carbohydrates in muscle[820] (see page 246).

Occurrence. Zymase occurs in various yeasts (*Saccharomyces*, lactose yeasts, etc.),[537] in certain fungi (*Aspergillus*, *Mucor racemosus*) and in bacteria (various cocci, bacilli and spirilla). Zymase has been demonstrated in various plants,[1169] in beets, peas, potatoes, in various leaves and flowers. The formation of lactic acid and alcohol in animal organs also leads one to suspect the presence of a zymase.

Preparation. The Buchner method[207,208] of preparation of yeast juice containing the endo-enzyme zymase, as well as other methods for the preparation of this enzyme have been described in a preceding chapter (see pp. 126–128).

Measurement. Since alcohol and carbon dioxide are formed as a result of the action of zymase, both, and particularly the latter, can be used in measuring the activity of the enzyme. Twenty cubic centimeters of yeast juice and 0.2 cc. toluol are placed in a 100 cc. Erlenmeyer flask. Eight grams of powdered cane sugar are gradually added and dissolved by shaking. This requires about one minute. The flask is closed with a stopper, which is connected

Oxidation of carbohydrates in muscle[928]


Glucose
+ H_3PO_4
Hexose phosphate
Labile hexose form + H_3PO_4
− 2 H_2O
Methylglyoxal
+ H_2O
Lactic acid
− H_2
+ H_2
Pyruvic acid
− CO_2
− CO_2
oxal-acetic acid
acetaldehyde
− 2H
+ 2H
+ H_2
− H_2
+ O
Malic acid
+ H_2O
− 2H
Ethyl alcohol
acetic acid
2 mol
− 2H
Succinic acid
Fumaric acid

through a sulfuric acid bulb (to dry the CO_2) with a burette for collecting the gas. The whole apparatus is weighed and incubated, at 22°C. for one, two, and three days and the gas measured. Since the toluol has a tendency to leak out, rubber connections are to be avoided, and only glass connections used. The amount of CO_2 evolved and the loss of weight serve as indices of the activity of the enzyme.

Properties of zymase. The optimal temperature is between 28° and 30°C., although 22°C. is less injurious to prolonged activity. In solution, zymase is destroyed at 40° to 50°C., but, in a dry state, it will withstand 85°C. The optimal reaction for zymase is pH 6.2 to 6.8.[407] Phosphates, and to a lesser extent nitrates, especially magnanese nitrate, urea and glycocoll have a stimulating effect upon zymase; fluorides, formaldehyde, peptone and acetone are injurious. Zymase is also sensitive to other enzymes especially to the tryptase of yeast, which will destroy the zymase in yeast juice in a short time. All substances, which favor the action of the proteolytic enzyme, will prove, therefore, injurious to zymase and vice versa. Active yeast juice, containing zymase, becomes inactive, after passage through a Martin filter or after dialysis through parchment paper, but if the filtered and unfiltered portions are combined, activity is restored. A co-enzyme is, therefore, filtered or dialyzed out. The co-enzyme can withstand a temperature of 100°C., is found in boiled yeast juice and is not enzymatic in nature. It is a salt of hexose-phosphoric acid, injured by 2.5 per cent H_2CO_3 at 25°C. and is completely destroyed by being heated at 130°C. for four hours. It is not affected by proteolytic enzymes but is destroyed by lipase because of the liberation of phosphoric acid. By treating macerated muscle tissue or yeast cells with distilled water, a dialyzable, thermostable substance, or co-enzyme, is washed out. The zymase is left in the solid part of the tissue, but it has lost its activity; this is restored by the addition of boiled muscle juice or yeast extract.[820]

Zymase acts upon three hexoses (*d*-gluclose, *d*-galactose and *d*-fructose) and their disaccharides; glucosides are not attacked, even if they have a glucose group, unless a specific enzyme is present which will break off this group. The formation of zymase by yeasts is stimulated by the presence of maltose, but injured by the presence of cane sugar, dextrose and fructose.[41,674] The treatment of yeast with galactose causes it to acquire the ability to ferment this sugar and reduces by half its power of fermenting glucose; when the yeast which has become adapted to galactose is again cultured on glucose media there is no loss of its galactose-fermenting power.[414]

2. Glycolase. Glycolase is an enzyme which decomposes glucose in animal tissues, with the formation of lactic acid. Claude Bernard in 1878 was the first to observe the disappearance of sugar when it was brought into contact with blood.[125,721] Lactic acid is formed in blood after glycolysis,[1196] and also if pieces of kidney, obtained aseptically, are immersed in a solution of glucose.[723] A

detailed study of the chemistry of the action of this enzyme may be obtained elsewhere.[923]

Occurrence. Glycolase is well distributed in the plant kingdom and is present in almost all of the organs of the animal body, especially in the blood. The glycolytic ferment is present only in the cellular elements of the blood and not in the plasma;[1033] the red blood cells were believed to be the chief carriers of glycolase in the blood,[823] but there is evidence, however, that the leucocytes contain considerably more of the enzyme than do the red blood cells. As a general rule, the greater the glucose content of normal blood, the more active is the glycolase,[1210] but this increase in glycolytic activity is not invariably present in normal blood in which the amount of sugar is high,[1196] nor is it found in the hyperglycemic blood of diabetic patients for the glycolase content is reduced,[310,721,1196] nor in the blood of dogs in which hyperglycemia has been produced by stimulation of the splanchnic nerves, asphyxia or by injections of epinephrin, for the glycolase content remains normal.[771] Insulin does not increase the glycolytic power of blood when added to it *in vitro*, nor when injected into normal animals,[356] but the glycolytic power of the blood of diabetic patients increases after treatment with insulin.[1196] It is possible that this increase in glycolytic action is caused by an indirect action of insulin on some ferment mechanism within the body. The presence of glycolase in milk is probably due to the presence of leucocytes.[1170]

Preparation. Animal zymase, or glycolase, is the most sensitive endoenzyme. No definite separation of the enzyme from the living cell has yet been accomplished. With every attempt to separate the enzyme, it is very readily destroyed. The enzyme is sensitive not only towards proteases, but also towards all antiseptics. By breaking up the cells by means of the Buchner pressure method, an active juice is obtained. The product can also be precipitated with alcohol or alcohol-ether.[1169-1171] The organ itself can also be treated by the acetone-ether method.

Measurement. The action of glycolase results in the decomposition of glucose and a quantitative measurement of the latter can, therefore, serve as an index of the activity of the enzyme. The process is carried out as follows:[1033] 50 cc. of blood are aspirated from the vein of an animal, under sterile conditions, into a flask with some glass beads, and defibrinated by shaking. (The presence of oxalate, to prevent coagulation, reduces the amount of glycolysis).[771] The blood is filtered through sterile gauze and 2 to 20 cc. portions are placed in two sterile flasks. One-half cubic centimeter of a sterile six per cent glucose solution is added to each with a sterile pipette. Both flasks are then shaken. Using the Bertrand[129,130b] method, the amount of glucose is determined immediately in one flask and, after five to six hours of incubation, in the other. Before the latter determination is made, the sterility of the mixture should be demonstrated. Sterile enzyme solutions should be employed but if antiseptics, such as toluol or chloroform, are used, they should not be in excess as they are injurious to glycolase. The glucose solution should be isotonic with blood, otherwise hemolysis will occur and the glycolase will be destroyed. If chloroform has been used as an antiseptic, all traces of it should be removed before the sugar determination is made. The determination of the carbon dioxide formed, as a result of the action of the enzyme, can also be used as an index of the activity of glycolase.[1171]

3. Glyoxalase. Glyoxalase acts upon methyl-glyoxal with the formation of lactic acid:

$$CH_3\cdot CO\cdot CHO + H_2O \rightarrow CH_3\cdot CHOH\cdot COOH$$

The enzyme is found in the liver and muscle of warm blooded animals, in nerves and in leucocytes. To prepare glyoxalase, finely ground tissues are extracted with ten volumes of physiological salt solution or distilled water for thirty minutes at 37°C., and then filtered through paper. The extracts thus obtained can be used directly as a source of enzyme. Glyoxalase is destroyed at 60°C. and is injured by acids. Pancreatic extract contains substances which act as anti-glyoxalases.[289a]

4. Carboxylase. Carboxylase is an enzyme which is capable of splitting off carbon dioxide from various aliphatic and aromatic keto acids.[830-832] It does not act upon carbohydrates, but only upon the intermediary substances containing a carboxyl group which are produced during the process of decomposition of complex organic substances including, of course, the substances possessing carboxyl groups which are formed by the action of zymase upon a carbohydrate. Carboxylase breaks down the carboxyl group, liberating CO_2:

$$\underset{\text{pyruvic acid}}{CH_3\cdot CO\cdot COOH} = \underset{\text{acetaldehyde}}{CH_3\cdot COH} + CO_2$$

$$\underset{\text{oxal-acetic acid}}{COOH \cdot CH_2 \cdot CO \cdot COOH} = \underset{\text{acetaldehyde}}{CH_3 \cdot COH} + 2\ CO_2$$

Actually 25 to 40 per cent of the theoretical amount of acetaldehyde is formed.

Occurrence. Carboxylase is present in fresh and dry yeast and in yeast juice; it also occurs in fungi.[343,346,1315]

Preparation. The methods are the same as those for zymase (see pp. 126–128).

Measurement. To demonstrate the presence of carboxylase, 15 cc. of M/10 pyruvic acid (0.88 gram or 0.7 cc. of freshly distilled pyruvic acid, in 100 cc. H_2O) and 1.5 cc. of 1 M K_2HPO_4 (17.4 grams K_2HPO_4 in 100 cc. H_2O) or 1.5 cc. 2 M $KC_2H_3O_2$ (19.6 grams $KC_2H_3O_2$ in 100 cc. H_2O) are placed in a test tube. Two grams of dry yeast or acetone-treated yeast are slowly added. The mixture is shaken and the test tube connected, by means of a glass tube, to a flask containing water. The test tube is placed in a beaker of water kept at 37°C. Carbon dioxide will be produced within a few minutes. The acetaldehyde can be collected by passage through a condenser. One cubic centimeter of the distillate is treated with 1 to 2 cc. of a dilute fresh sodium nitro-prusside solution and 1 to 2 drops of 5 per cent diethylamine or piperidine. The presence of acetaldehyde will be indicated by a deep blue color.

For a quantitative estimation of the acetaldehyde, the complete distillate is treated with a clear filtered solution of an acetic acid solution of *p*-nitrophenylhydrazine with which acetaldehyde forms a precipitate (acetaldehyde-*p*-nitrophenylhydrazone). After one hour, the precipitate is filtered through a weighed Gooch crucible, dried at 90°C. and weighed again. One hundred and seventy-nine grams of the precipitate correspond to 44 grams of acetaldehyde which is the amount formed by the decomposition of 88 grams of pyruvic acid.[1298] If the alkali salts of acids are used, a buffer must be added to prevent excess alkalinity; boric and arsenic acids are suitable for this purpose.[882]

The formation of carbon dioxide can also be demonstrated by mixing thoroughly 2 grams of yeast with 12 cc. of a 1 per cent solution of pyruvic or oxalacetic acid, placing the mixture in a proper apparatus and collecting the carbon dioxide. By introducing a small amount of potassium hydroxide into the tube containing the CO_2, the purity of the gas can be established.

5. Fumarase. Fumarase hydrolyzes fumaric acid to malic acid. The reaction is reversible; equilibrium occurs after about two-thirds of the fumaric acid is converted into malic acid:

$$\mathrm{HOOC\cdot CH{=}CH\cdot COOH + H_2O \rightleftarrows HOOC\cdot CH(OH)\cdot CH_2\cdot COOH}$$

All animal tissues contain this enzyme.[98,290,372] Ten grams of liver or about 33 grams of muscle tissue are required to hydrolyze 2 grams of fumaric acid to the equilibrium point in 15 minutes at 40°C. The concentration of fumarase is parallel to that of succino-oxidase, which oxidizes succinic acid to fumaric or malic acids. Fumarase is soluble in water, is inactivated by alcohol, by trypsin and by heating to 52° to 54°C. It acts best at a neutral reaction and at 40°C.

6. Carboligase. Carboligase is a synthesizing enzyme binding carbon atoms and found in yeasts:[887]

$$\mathrm{C_6H_5\cdot CHO{+}CH_3\cdot CHO = C_6H_5\cdot CHOH\cdot COCH_3}$$

7. Glycerolase. The active enzyme which digests glycerol has not been described but glycerol fed to phlorizinized or depancreatized dogs is converted into glucose.[242,282,763]

CHAPTER XIV

CATALASE

Catalase, like inorganic colloidal catalyzers, decomposes hydrogen peroxide into water and inactive molecular oxygen:[744]

$$2H - O—O - H = 2H_2O + O_2$$

It is not an oxidizing enzyme and must be distinguished from peroxidase, which activates the oxidizing action of peroxides. Peroxides, other than H_2O_2, are not attacked by catalase. H_2O_2 is sometimes considered as a constant intermediary product of the hydration of oxygen.[1270a] This toxic H_2O_2 is rapidly decomposed either by an excess of hydrogen or by the enzyme catalase.

Occurrence. Catalase is present in all living tissues, including various animal organs, blood (see p. 79), secretions and excreta, plants and microörganisms. The addition of catalase prevents the destruction of xanthine-oxidase by the H_2O_2 formed during the oxidation of purine bases.[329]

Preparation. Catalase may be prepared by mixing defibrinated blood with about ten volumes of water saturated with carbon dioxide and allowing the mixture to stand ten hours.[789,1116] The liquid is then filtered off and added to 1½ times its volume of 95 per cent alcohol. The resulting precipitate is extracted with water, filtered and reprecipitated with 3 volumes of alcohol. The light brown precipitate is then dried in a desiccator, ground and extracted with water. The enzyme content of this extract may be increased by concentration in vacuo.

Another method of preparation consists in centrifuging fresh defibrinated blood and then washing the red blood cells three times with physiological salt solution. They are then centrifuged and hemolyzed in distilled water and ether. After the addition of alcohol at 10°C. or less, the hemoglobin is allowed to crystallize out and is filtered off. The mother liquor is rich in catalase and is active for a long time.[1301] The enzyme may also be extracted from fat and various organs, as well as from plants and microörganisms, by grinding the material with sand and chloroform water. The resulting extract is then precipitated with alcohol and dried, or with ammonium sulfate, and then dialyzed. Chloroform can also be used for the purification of blood catalase, since hemoglobin is precipitated by chloroform. The enzyme can also be purified by adsorption with $Ca_3(PO_4)_2$ and extraction with Na_2HPO_4 solution.[1205] A very active catalase may be obtained from the liver by grinding the latter at a low temperature, extracting with water, precipitating with 2 volumes of alcohol, filtering, drying, again extracting with water, precipitating with alcohol and drying.[93] One gram of this preparation decomposed 4 kgm. of H_2O_2 in ten minutes at 15°C. The catalase may also be adsorbed by tricalcium phosphate and removed with disodium phosphate. Cultures of bacteria, grown in media of known composition may be used as a source of catalase; the enzyme can be precipitated from the medium by ammonium or magnesium sulfate, or with mercury chloride, or by 2 volumes of methyl alcohol.[606] The enzyme is extracted from the alcohol precipitate with water and the latter removed by drying at a low temperature.

Measurement. The presence of catalase can be demonstrated qualitatively by adding a few cc. of H_2O_2 to the solution under investigation and shaking. The formation of bubbles indicates the presence of catalase. For the quantitative measurement of catalase, three methods are available: (1) the permanganate, (2) the iodometric and (3) the direct measurement of the oxygen evolved.

The *permanganate* method is based upon the fact that H_2O_2 decomposes permanganate, in the presence of sulfuric acid:

$$2KMnO_4 + 5H_2O_2 + 4H_2SO_4 = 2KHSO_4 + 2MnSO_4 + 8H_2O + 5O_2$$

5 to 10 cc. of the catalase solution are added to 20 cc. of 1 per cent H_2O_2, in a 200 cc. Erlenmeyer flask, and allowed to stand for two hours at room temperature. Ten cubic centimeter of 10 per cent H_2SO_4 and 30 to 40 cc. of H_2O are then added and the mixture titrated with N/10 $KMnO_4$ until a permanent rose color is formed. If the first drop of permanganate solution causes a red coloration, either the amount of H_2SO_4 is insufficient or all of the peroxide has been decomposed. A control flask is prepared, using 5 to 10 cc. of distilled water instead of the catalase solution and the titration value of the control is subtracted from that of the enzyme mixture. The amount of $KMnO_4$ solution used is an index of the concentration of catalase. If 0.37195 per cent $KMnO_4$ is used for titration, the actual amount of H_2O_2 decomposed can be readily calculated, for 1 cc. of this solution is equivalent to 0.002 gm. H_2O_2.[1298]

This method has been improved by the following modification:[1205] 90 cc. of M/150 H_2O_2 and 10 cc. of a mixture of equal parts of M/15 Na_2HPO_4, and M/15 KH_2PO_4 are placed in each of 3 flasks which are then placed in an ice bath at 2°C. The reaction of these solutions is pH 7.0. To one flask, 10 cc. of the enzyme solution are added, to the second, 5 cc. of the enzyme solution and 5 cc. of the buffer mixture described above and, to the third, 10 cc. of the buffer mixture as a control. After a definite period of incubation at 2°C., 20 cc. of 20 per cent H_2SO_4 are added to each flask and the contents titrated with N/20 $KMnO_4$ until a permanent rose color is formed. The titration value of the control is subtracted from those of the enzyme mixtures.

The *iodometric titration*[625] is based upon the fact that H_2O_2 liberates free iodine from KI. The amount of this iodine can be determined quantitatively by titration with thiosulfate solution. Two cubic centimeters of the enzyme solution (in the case of blood, 1 cc. is diluted to 500 cc. with physiological salt solution and 2 cc. of this dilution used) are mixed with 20 cc. of a 1 per cent H_2O_2 solution, and incubated at room temperature for two hours. Ten cubic centimeters of dilute H_2SO_4, 10 cc .of 1 per cent KI solution and some dilute starch paste are then added and allowed to stand for one hour. The undecomposed H_2O_2 liberates free iodine from the KI solution, and the solution becomes blue. The mixture is then titrated with a standard sodium thiosulfate solution, and the amount of undecomposed H_2O_2 can be calculated. The "catalase number" of Jolles[625] is the number of grams of H_2O_2 which will be decomposed by 1 cc. of blood. The values for human blood usually vary from 18 to 30 (average 23); in pathological cases the value is lower. This method is unsuitable for measuring bacterial catalase because bacteria themselves liberate iodine from KI.[633]

The *volumetric* determination is based upon measurement of the oxygen formed from a 1 per cent solution of H_2O_2 under definite conditions (18°C. for ten minutes, for example).[93] A convenient apparatus has been described for this purpose.[37]

Conditions which affect the activity of catalase. Catalase is active even at 0°C. and its action is more or less constant between 10° and 40°C.[829] At 45 to 55°C. its activity is diminished and at 65°C. the enzyme is destroyed.[1205] Catalase is an ampholyte, with an isoelectric point at pH 5.37; the anion and the undissociated part are considered to be the active part of the enzyme.[829] The optimal reaction is near neutrality, the exact point depending upon the length of incubation; the activity diminishes on both sides of the neutral point.[606,850,1158] For very short periods of incubation, however, the optimal reaction is alkaline, being pH 7.5 in tests which last 10 minutes, pH 8.0 in those lasting five minutes, and pH 10.0 in those of two minutes.[151] Prolonged exposure to reactions of pH 4.5 and 8.5 tends to destroy catalase. Salts usually have an injurious effect upon catalase, but in various degrees, as shown by the following series:[1078]

$$CNS > ClO_3 > CN > NO_3 > OH > I > Br_7CO_3 > Cl > B_4O_7 > F > HPO_4 > SO_4$$

Secondary phosphates increase the activity of catalase because they stabilize the hydrogen-ion concentration. Chloroform and other antiseptics are of low toxicity; these may even activate the catalase of certain cells, as for example yeast suspensions. However blood cells are activated only by warming (to a maximum of 57°C.) and cooling rapidly.[397,402] An excess of H_2O_2 (above 0.2 molar) brings about a reduction in the action of the enzyme.[850,1078,1116]

As to the kinetics of the action of catalase, it has been shown that:[850,903] (1) The rate of formation of oxygen is independent of the concentration of the peroxide, when the latter is greater than 0.1 molar; (2) the rate of decomposition of the peroxide is proportional to the concentration of the catalase present and (3) the catalase undergoes spontaneous monomolecular decomposition during the reaction; the decomposition is independent of the concentration of catalase and is inversely proportional to the original concentration of peroxide up to 0.4 molar. If x is the amount of O_2 liberated in time t, A the total amount of O_2 liberated and K the inactivation constant of the enzyme,

$$K = \frac{1}{t} \log \frac{(A)}{(A - x)}$$

A detailed review of the chemical nature and physiological rôle of catalase may be obtained elsewhere.[93,850]

D. PRACTICAL APPLICATIONS OF ENZYME ACTIVITY

CHAPTER XV

USES OF ENZYMES

1. USE OF ENZYMES IN THE TEXTILE INDUSTRIES. *a. Diastatic enzymes.* Diastatic enzymes, which convert starches into dextrins and finally into sugars, are employed in various processes of the textile industry, for example: in the sizing of warps prior to weaving, in the preparation of a soluble starch as a base for finish, in the stripping and degumming of sized cloth, in the treatment of goods previous to mercerization, in the treatment of cloth and fiber previous to bleaching, and in the preparation of thickness for printing colors.[789] In preparing the size from wheat or potato starch, a thick mixture of the starch and water is poured into boiling water and stirred constantly. The paste which is formed is cooled to 70°C. and the diastatic enzyme, in the form of malt extract, pancreatic gland preparations, *Asp. oryzae* diastase or bacterial diastase is added. Digestion of the starch is carried to a point at which the paste becomes liquefied, thin and transparent. The enzyme is then inactivated by boiling and various binding or stiffening agents are added to produce size of the proper consistency.

Diastatic enzymes are also used to convert starch into soluble starch, or into a mixture of soluble starch, dextrins and maltose, for the purpose of finishing. Different thicknesses of starch can be obtained by varying the quantity of diastase and the time of its action.

To remove the filling of a heavily finished cloth, the goods are steeped or passed through a solution of the enzyme. The material is subsequently passed through boiling water and dried. In the mercerization of cotton goods a full gloss can be obtained by stripping the cloth of all size by means of diastatic enzymes. After printing, the excess of starchy material can also be removed by the same method. In laundries, diastatic enzymes can be used for removing the starch from collars and other goods so as to facilitate subsequent cleaning. Finally they may also find an application in the sizing of paper.[1106]

b. Pectic enzymes. Linen, flax and cotton goods contain pectic substances which consist of mixtures of pectins, proteins and starches, and are usually classified under the name of gums. The treatment of these textiles in a warm bath containing a pectic enzyme renders the pectins soluble and facilitates their subsequent removal by boiling. Most of the common diastase preparations can be used for this purpose as they usually contain pectinase and pectase. The process of "retting" of flax consists in the dissolution of the pectins, which bind together the cellulose fibers; it is usually performed by exposing the flax to various bacteria and fungi, which carry on this process by means of specific enzymes. At present, living organisms must be employed in the industrial process of retting, as in the case of the Rossi and Carbone processes in which bacteria are employed, or the dew process in which fungi are chiefly used.[114,1066] However, if a sufficiently strong pectic enzyme (pectase and pectinase) could be obtained, the process of "retting" could be accomplished in a much simpler manner.

c. *Proteolytic enzymes.* When cotton goods are steeped in crude diastatic enzyme mixtures (see p. 259) the proteolytic enzymes which are always present act incidentally upon the proteins of the cloth, but, in addition, there are certain distinct processes in which the latter enzymes have a specific application, especially in the degumming of silk goods,[1024] as silk fibers are bound together by means of a specific protein belonging to the gums. Degumming is normally accomplished by boiling in soap, but preliminary treatment with a proteolytic enzyme effects a great saving in the amount of soap. Enzymes that are tryptic in nature can be readily used for this purpose. It is possible that the Kier-boil process, which consists in boiling cotton goods in an alkali solution, in order to remove the gums, starches, proteins and pectins may eventually be replaced by an enzymatic process. This would involve treating the cotton goods first with a softening agent and then with a mixture of diastatic, proteolytic and pectic enzymes, possibly followed by a soap wash.

2. Use of Enzymes in Tanning. During the various operations through which hides pass in the process of their transformation into leather, enzymes, especially proteases and lipases, play an active part. To prepare leather from a raw hide, the epidermis and subcutaneous tissue must be removed by the processes of unhairing and "bating" of the skins. The object of the latter operation is to prepare the unhaired skins for tanning and it consists in the removal of elastin from the skin.[687,1290,1292] It has been also suggested recently

that one of the important functions of bating consists in the removal of keratose from the skins by hydrolysis with enzymes.[1292a] This process of "bating" or "puering" is carried out by means of a proteolytic enzyme, such as trypsin, in conjunction with a deliming agent such as ammonium chloride.[1031] The operation of unhairing consists in allowing the skins to swell in dilute caustic soda. The swelling is removed by sodium carbonate and a proteolytic enzyme is then added to loosen the hair sufficiently so that it can be easily removed.[572] The process, which appears to have a number of advantages over the old system of liming, is carried out as follows: The skins are first soaked for one day in dilute sodium hydroxide and then transferred to a solution containing the enzyme and ammonium salts or sodium carbonate.[572] The use of the proteolytic enzymes of fungi (*Asp. oryzae, Asp. wentii*, and Penicillia) has been suggested for this purpose.[720a] After treatment for twenty-four hours the hair is loosened and can be easily removed. It is possible that the unhairing process which is usually carried out at 25°C. and pH 7.5, is due not to the enzyme, but to proteolytic bacteria.[1291] Up to 40°C. trypsin has no appreciable action upon the fresh hide substance or collagen, if the skins have not been previously treated with acid. If they have, the hide substance is much more readily attacked by trypsin. Acid swelling is, therefore, not practiced in the "beam-house," a department of the tannery where the skins are kept before tanning. If the skin has previously been soaked in dilute sodium hydroxide, pancreatin has a decided unhairing action at 40°C. The effectiveness of the enzyme as an unhairing agent apparently depends upon the previous swelling of the skin, regardless of whether this has been produced by an acid or an alkali. Formerly the unhaired skins were kept in a warm infusion of dog or chicken manure until they were softened. A fresh infusion of manure is practically free from enzymes but, after fermentation for twenty-four to forty-eight hours, five different enzymes usually develop: two proteolytic enzymes—one resembling pepsin and the other trypsin—; rennet; diastase; and a lipase.[1013] Of these, lipase acts on the fats in the skin while the proteolytic enzymes act on the elastin fibers. The reaction at which the elastin is removed by enzymes is pH 5.5 to 8.5. There is also another application of enzymatic activity in tanning, namely the use of *Asp. niger*, which produces tannase very actively, in the fermentation of gall nuts. Further information on the hydrolysis of collagen by trypsin and on the mycology of tanning may be obtained elsewhere.[1198]

3. Other Industrial Applications of Enzymes. Wherever fats, starches, or proteins are to be removed with the least damage to the material which contains them, enzymes should be used since they are relatively inexpensive and usually effective. For example, in freeing starch from proteins, the mixture can be treated with *B. putrificus* or any other strongly proteolytic bacterium or enzyme. The greatest difficulty in this connection is that certain enzymes, especially pectases and lipases, as yet cannot be manufactured cheaply enough to offer a substitute for the acid and alkali treatment which in itself is often injurious to the fabric or other material which is to be purified. In the manufacture of glycerin and fatty acids, castor oil bean lipase is often utilized. Invertase plays an important rôle in sugar manufacture.[292] Proteolytic enzymes can be used in the stripping of the gelatin coating from photographic plates and motion picture films, for the recovery of the silver salts.[296a]

4. Use of Enzymes in the Preparation of Beverages. *a. Rôle of enzymes in brewing.* i. *Malting process.* Malt is commonly prepared from barley; the barley is first soaked in water for about forty-eight hours; during this time it absorbs 40 to 50 per cent of its weight of water. It is then spread out in a layer several feet in thickness until germination begins and the temperature begins to rise. The material is then spread over the floor and mixed from time to time to provide proper aeration until the young plant is about three-fourths of the length of the kernel. Germination of barley can also be carried out in drums or compartments, in which the grain is mechanically mixed and the moisture and temperature factors controlled. The enzymes—cytase, diastase, protease, lipase, phytase, and oxidase,—take an active part in the transformation of the insoluble constituents of the grain, allowing the embryo to draw upon the soluble food materials for its growth. By regulating the temperature and air supply so that the development of the embryo is not retarded, a large amount of enzyme accumulates in the germinating grain, without any great loss of carbohydrates and proteins through growth. As a rule, about one week is required for the whole process of malting. This product, which is called green malt, is then subjected to a kilning process which interrupts germination, removes excess moisture, and produces the characteristic flavor. It is first warmed at low temperatures, to drive off some of the moisture, and then heated to a high temperature, ranging from 80° to 98°C. The heating and kilning are usually done by direct fire gases in large kilns. Some of the enzymes, such as cytase, are completely

destroyed while others, like diastase (especially the amylolytic fraction), are not affected to any great extent.[1246] The dried malt is then ground.

ii. *Mashing process.* This consists in incubating the malt so that the enzymes which are present can digest the insoluble material which is contained in the malt or in the flour or other ground ungerminated cereal added to it. The ground malt is mashed with water at 40° to 50°C. until the mixture or "wort" contains the dissolved substances in desired quantities and proportions. By varying the temperature and length of incubation the relative amounts of dextrins and maltose formed are controlled; these in their turn affect the alcohol content of the beverage. (In distilleries, a high grade malt is employed; this is obtained by careful regulations of the kiln-drying, so as to destroy as little of the diastase as possible. The criterion for good malt is that one part should convert, in addition to its own starch, eight to nine parts of corn, previously gelatinized by cooking.[1246] When the mashing process is completed, the "wort" is separated and boiled with hops; all enzymes are thereby destroyed and the albumins coagulated. The "wort" is then cooled and inoculated with yeast in order to produce alcohol. The rôle of enzymes in the formation of alcohol from carbohydrates ("fermentation") has been discussed on page 244. A detailed study of alcoholic fermentation may be obtained elsewhere.[524, 886a]

iii. *Clarification of beer.*[1246] In view of the fact that some of the proteins of the original grain have been only incompletely decomposed during the malting and mashing process and that the resulting liquid often becomes turbid on standing in a cold place, the addition of proteolytic enzymes, such as pepsin, papain or bromelin, has been advocated in order to decompose the proteins completely.

b. Enzymes in the distillery. Enzymes are extensively used in the industrial manufacture of alcohol; the particular one employed depends upon the material used as the starting point. In the case of starchy products, diastase, maltase and zymase are the active enzymes, in the case of molasses, invertase and zymase are important. Both invertase and zymase are produced by yeast.

c. Preparation of various other beverages. The most common beverage used throughout Russia is kvass. This is usually prepared by mixing 170 pounds of rye malt, 160 pounds of barley malt and 160 pounds of rye flour, pouring the mixture into boiling water, stirring, and allowing it to stand for nine to ten hours. The mixture is then diluted with 80 pailfuls of boiling water and allowed to stand another eight hours. Five pounds of peppermint as a flavoring substance and $\frac{3}{4}$ pound of yeast are added and the mixture incubated for two to three days. The resulting beverage contains from a trace to 2.6 per cent of alcohol, together with lactic and acetic acid. The preparation of various beverages from milk, such as kefir, koumis, etc., depends upon the activities of certain bacteria and their enzymes.

5. Use of Enzymes for the Clarification of Fruit Extracts. *a. Clarification of pectin preparations.* The process of preparing pectin from fruit residues consists in soaking apple pomace in water to extract the soluble sugars. The liquor is removed and can be used for making vinegar. Water is again added to the residual fruit and the pectin is extracted by boiling, or autoclaving at a pressure of 10 to 20 pounds. The liquid is then pressed out. The pectin solution thus obtained is turbid, due largely to the presence of a small amount of starch and protein. These are removed by treatment with a diastatic enzyme preparation which also contains a protease. *Asp. oryzae* diastase is preferable to malt diastase for this purpose since it completely hydrolyzes the starch and is more active at the acidity which exists in pectin preparations. The pectin solution is then filtered and concentrated in a vacuum pan. The pectin may also be obtained by precipitation with suitable agents. Further information on the preparation of pectin from apple peelings and cores may be obtained elsewhere.[228a] Citrus fruit juices may be clarified by removing the proteins by digestion with proteolytic enzymes which are active at an acid reaction.

b. Clarification of syrups. In the process of preparing sorghum syrup, starch is largely responsible for the jellying and for the difficulty with which this syrup filters.[1135] Preliminary removal of this starch with diastase prevents subsequent jellification and facilitates filtration. In general, all fruit extracts, syrups, beverages and similar solutions which become cloudy on standing, or which are filtered with difficulty because of the presence of starches and proteins may be readily clarified by the use of the proper enzymes. The reaction of the juice as well as the nature of the substances causing the turbidity are of prime importance. In the case of starches, *Asp. oryzae* and malt preparations, preferably the former, are to be employed if the reaction is acid, and pancreatic preparations if the reaction is alkaline (unusual in the case of fruit juices). For the removal of proteins, *Asp. oryzae* preparations, papain and bromelin, can generally be used. Pepsin can also be used in acid solutions and trypsin in alkaline solutions.

6. USE OF ENZYMES IN FOOD PREPARATIONS. *a. Bread making.* Diastatic and proteolytic enzymes play an important part in bread making.[269,1065] The panary fermentation which is caused by yeast depends largely upon the presence of sugars produced by the diastase of the flour. Diastatic enzymes are therefore among the most important factors which make up the "strength" of flour. The optimal temperature for the diastatic activity of malt flour is 65°C. The optimal reaction for the activity of malt diastase is pH 4.3 and of flour diastase pH 4.7. The increased temperature at "proofing," together with the increased hydrogen ion concentration during the process of bread fermentation (from pH 6.0 to 5.0), combine to increase the action of diastase during the later stages of fermentation. The addition of diastatic enzymes to a dough causes an increase in the amount of reducing sugars which later disappear as a result of the action of the yeast. The use of malt flour and malt extract in doughs increases the volume of the bread and produces a superior loaf. To prepare leavened bread, the addition of the following ingredients to every 100 pounds of flour has been recommended:[676] 8 ounces of enzyme mixture and 8 ounces of a mixture consisting of 10 parts of NH_4Cl, 25 of $CaSO_4$, 0.3 of KBO_3, 25 of NaCl and 40 of flour.

b. Cheese making. The preparation and ripening of various cheeses depend chiefly on the action of enzymes, especially rennet and proteolytic enzymes. These are either formed by the activities of microörganisms or are added with the rennet used to coagulate the milk. In making cheese, skimmed or unskimmed milk is first coagulated by the addition of a standard fresh liquid extract of rennet, although in some cases, macerations of dried calf stomach linings prepared in an acid medium are used. In the latter, heat-resistant bacteria are also introduced. (The qualities required of rennet are: uniform strength, stability and freedom from pepsin, fermentable organic matter, antiseptics, and acids.[965]) The rapidity of coagulation depends on the amount of rennet used, the reaction of the liquid, and the temperature at which coagulation is carried out. These factors also influence the consistency of the curd, producing soft, medium, firm, elastic or oily curds. The curd is then separated from the whey, a process which is hastened by heating. Draining and pressure are used to reduce the amount of whey to the proper proportions. Salting will modify the flavor, control the types of developing organisms and aid desiccation.

The final cheese is produced from the curd as a result of the action of the various enzymes of the microörganisms present. Among the enzymes concerned in the ripening of cheese are: (1) pepsin which is usually present in the rennet preparations; (2) various proteolytic enzymes present in the milk or secreted by the various microörganisms developing on the surface or within the curd; (3) various desaminases and desamidases, which cause the formation of volatile acids; (4) lipase and other esterases which act upon the butterfat and various esters; (5) carbohydrases, especially lactase, formed by the lactic acid bacteria and various fungi; (6) lactacidases which produce lactic acid from the monosaccharides present, and (7) oxidases which act upon various organic acids.[338,363]

The chemical action of rennet upon casein is closely related to that of pepsin;[1215] its hydrolytic action, which results in the formation of albumoses and peptones, is intensified by the increase in acidity which develops during the ripening of the cheese.[167] The cheese aroma is due to amino acids and ammonia which are formed by a specific decomposition of the fats and proteins, brought about by the activities of certain specific bacteria,[916a] and to some extent by their enzymes.

c. Production of syrup from sweet potatoes. Sweet potato syrup can be manufactured by the conversion of the starch of sweet potatoes by means of malt.[480] This is accomplished by adding 1 part of malt to 500 parts of potatoes and allowing the mixture to stand forty-five minutes at 66°C. The crude or filtered syrup can then be concentrated in an evaporator.

d. Production of invert syrup from cane sugar. Various processes have been devised for the utilization of the inverting action of invertase upon sucrose in the manufacture of golden syrup, cane and sorghum syrups, maple syrup, maple cream and the fondant types of confectionery.[940] The inversion of the sucrose increases the solubility of the total sugars, thus increasing the density of the syrup; it controls the crystallization of the sucrose, modifies the flavor, and retards the drying of the fondant confections because of the hygroscopic character of the fructose. A discussion of the use of invertase in the confectionery industry may be obtained elsewhere.[938]

e. Manufacture of soy sauce and related products. The proteolytic and diastatic enzymes of the fungi, *Asp. flavus* and *Asp. oryzae*, are utilized in the Orient, especially in Japan, for the manufacture of various food preparations of which *shoyu-sauce*, *miso* and *saké* are the most important. In preparing soy (or shoyu) sauce from soy beans and wheat, the former are soaked in cold running water for about twenty hours and then cooked until they are soft, either by autoclaving at a pressure of fifteen pounds for fifty minutes or by boiling in an open kettle for a longer period. The beans are then drained and cooled as rapidly as possible. The wheat is roasted uniformly in large iron pans and then crushed. The powdered wheat and the sterile cooked beans are mixed in the proportion of 1:2 or 1:3 and inoculated with spores of *Asp. flavus* (tane-koji). The mass is mixed, distributed in flat trays, and incubated at 25° to 30°C. for two to three days. The trays are arranged to allow proper aeration. After eighteen to twenty-four hours the temperature in the trays begins to rise and a heavy development of mycelium appears. The mixture is then stirred so that the material from the bottom of the trays is brought to the surface. The temperature should not rise above 35° to 40°C. After forty hours' incubation, the fungus growth becomes yellow and is called koji, or shoyu-koji. This product is then placed in sodium chloride brine at 20° to 22°C. The resulting mash or shoyu-moromi is next inoculated with yeast (*Zygosaccharomyces* sp.), incubated and stirred daily. During this process of ripening, which lasts about one year, various proteolytic and diastatic enzymes of the fungus take an active part.[254,1072a] The soy sauce thus obtained contains about 30 per cent of dry material consisting of sodium chloride, ammonia, amino acids, purine bases, free acids (including lactic), and alcohol.

Miso is prepared by mixing cooked soy beans with koji (or bran covered with the growth of Aspergillus), adding varying amounts of salt and allowing the mixture to ripen. The strength of brine, variety of fungus and nature of the flavoring substance used influence the type of product formed.

Saké, a beverage containing 12 to 15 per cent alcohol is prepared from steamed rice which has been transformed into sugar by the strong diastatic enzyme of koji, or by the growth of *Asp. oryzae* on the rice. The sugar-containing mass is then fermented by means of yeast.

Taka-diastase, Kashiwagi-diastase, Digestin, Protozyme, Polyzyme, and many other commercial preparations are merely trade names for the enzyme prepared by growing *Asp. oryzae* upon bran. Wheat or rice bran is steamed for one or two hours, cooled to 25° to 30°C., then inoculated with a suspension of spores of *Asp. oryzae.* The inoculated bran is spread on trays which are placed on specially prepared racks. The doors of the chamber are then closed and the temperature is raised by means of steam to 30°C. After twenty to twenty-four hours, the spores germinate and the temperature in the trays begins to rise. The doors are then opened and air is blown through the chamber to lower the temperature. After forty to forty-eight hours, the bran is completely covered with a cottony mycelium. The mass is now removed from the trays, placed in special percolators and the enzyme extracted by percolation with 1 to 2 volumes of water for every volume of the bran material. The liquid may be filtered through infusorial earth or may be merely strained. If the enzyme is to be used for industrial purposes, as in the case of desizing of cotton goods, it can be preserved by means of a disinfectant such as thymol, sodium fluoride, phenol or cresol; if it is to be used for food or medicinal purposes, it is concentrated in vacuo at 30° to 40°C. to a syrupy mass, or it may be precipitated by 70 per cent saturation with alcohol, washed, and dried. The organism may also be grown on the steamed bran in a slowly rotating drum, similar to a malting drum. A convenient apparatus has been devised for the manufacture of the enzymes of microörganisms.[156]

7. USE OF ENZYMES IN POULTRY FEEDING. The addition of *Asp. oryzae* diastase or various commercial preparations of the organism grown on bran to poultry diets increases the rate of growth of young chicks.[556] After ten weeks' growth, the control chicks weigh 1.35 pounds, while those which receive 1 per cent of the enzyme preparation in their food weigh 1.50 pounds and those which are fed 5 per cent of the enzyme weigh 1.75 pounds. After a period of twenty weeks, the controls weigh 2.65 pounds, those fed 1 per cent *Asp. oryzae* diastase 2.80 pounds and those receiving 5 per cent of the enzyme 3.24 pounds. There is also an increase in the number of eggs laid by the chickens receiving enzyme in their mash.

8. ENZYMES IN THE RIPENING OF TOBACCO. The fermentation of tobacco is probably not microbial but enzymatic in character for when the temperature is increased to 63°C. in the curing process, the tobacco becomes practically sterile and yet fermentation goes on.[667] By developing a process of "forced fermentation," the length of time required has been reduced to twelve hours.

9. ENZYMES IN THE FERMENTATION OF CACAO. A number of enzymes are concerned in the fermentation of cacao which precedes the roasting process; diastase is the chief of these. By the enzy-

matic process about 13 per cent of the cacao seed is converted into "sweatings" which consist largely of reducing sugars with some mucilage and pectin. The temperature of the process reaches 47° to 49°C. in three days. At this temperature the cacao seed are killed while the enzymes exert their maximal activity. Among the other enzymes active in this process, are proteolytic enzymes, raffinase, invertase and oxidase.[193,668] The part played by the enzymes of *Asp. ochraceus* in the fermentation of coffee by this organism is still undetermined.[1197a]

10. USE OF OF ENZYMES AS LABORATORY REAGENTS. *a. Soy bean urease.* The accurate estimation of urea in animal fluids such as urine and blood can be readily accomplished by the use of urease.[784] The addition of ground soy beans or the urease extracted from soy beans to fluid containing urea results in a quantitative transformation of the latter into ammonia. The amount of ammonia is then determined either by direct titration, or by the titration of a standard acid solution into which the ammonia has been aerated.

b. Analytical determination of starches and sugars by means of enzymes. i. *Starches.* The analytical determination of starches in plant products (leaves, etc.) by the Official Method of transformation of the starch into sugar by boiling with acid presents several difficulties, especially those due to the loss of a certain amount of the dextrose. The use of the purified enzyme of *Asp. oryzae* (Takadiastase) has therefore been suggested for this determination.[301] The method is based upon the assumption that the enzyme converts starch into maltose and dextrose, free from dextrin. To determine the amount of starch in plant materials all plant enzymes are first destroyed by immersion in boiling 95 per cent alcohol containing some ammonia. The sugars are then extracted with boiling alcohol for eighteen to twenty-four hours in a Soxhlet apparatus. The pressed material is next dried, ground, and gelatinized in 200 cc. of water at 100°C. This solution is cooled to 38°C., 0.1 gram of Takadiastase and 2 cc. toluol are added, and the mixture is incubated for twenty-four hours. The solution is then heated to destroy the enzyme, filtered through paper, and the precipitate washed. Basic lead acetate is used to precipitate the tannins. The lead is then precipitated with solid sodium carbonate. One aliquot portion of the filtrate is used for the reduction of Fehling's solution, and the rotation of another portion determined in a 400 mm. tube. From the values x and y obtained by reduction and polarization, the amounts of dextrose and maltose present can be calculated and the original quantity of starch determined. However, later studies seem to point to various limitations in this method.

ii. *Sucrose and raffinose.* Invertase is used for the determination of sucrose;[909,933] top yeast containing invertase and bottom yeast containing invertase and melibiase are employed for the analytical determination of raffinose.[99,584] The latter method is based upon the fact that invertase converts raffinose to melibiose and fructose, while melibiase further hydrolyzes melibiose to galactose and glucose. The solution containing a mixture of sugars is first treated with invertase from top yeast and then with invertase and melibiase from bottom yeast. The degree of polarization is determined after each treatment and the difference in rotation indicates the quantity of raffinose in the mixture. This method has been modified and applied to factory control processes:[939] A weighed quantity (39 or 78 grams) of material is placed in a 300 cc. volumetric flask, 2 to 10 cc. of 55° Brix basic lead acetate solution are then added and the volume made 300 cc. at 20°C. The mixture is filtered through paper and the first 25 cc. of the filtrate are discarded. Two-tenths to 0.4 gram of $NH_4H_2PO_4$ are added to the filtrate to precipitate the lead and a small amount of infusorial earth to aid filtration. The mixture is again filtered and the first 25 cc. of the filtrate are discarded. To one 50 cc. portion of this filtrate, 5 cc. of top yeast extract (invertase) are added and to another portion 5 cc. of bottom yeast extract (invertase and melibiase). Hydrolysis of the sugars is allowed to proceed overnight at about 20°C. If the solutions are to be kept for a longer period of time, a small amount of toluol is added. The solutions are then diluted to 100 cc. and the polarization determined at 20°C. The number of grams of sucrose and raffinose in the product are then determined by specific equations in which R is the percentage of raffinose, S the percentage of sucrose, A and B the polarization after hydrolysis with top and bottom yeast extracts, and P the direct polarization in a 20 cm. tube at 20°C. of a solution containing 26 grams of a mixture of pure sucrose and raffinose per 100 cc. expressed in degrees Ventzke:

$$R = 1.354\,(A - \mathrm{B})$$

$$S = \frac{P - 2.219A - 1.219B}{1.3213}$$

iii. *Other applications.* The fact that certain enzymes act upon specific substances (emulsin on β-glucosides, maltase on α-glucosides, etc.) can be utilized for the determination of the presence of these substances. Enzymes can also be employed in the study of the action of poisons.[1036] The rôle of oxidizing enzymes in the Manoilov test of sex differentiation in animals and plants still remains unexplained.

11. Enzymes as Therapeutic Agents in Clinical Medicine. The body contains a variety of enzymes and anti-enzymes and the suggestion has been made that a disturbance in balance between these substances may lead to pathological processes. If the amount of anti-trypsin is reduced it is conceivable that the serum and tissue protease might digest body proteins, and also if protein splitting with its resulting formation of toxic split-products is a factor in the intoxication of acute or chronic conditions, the detoxication by means of ereptase and similar enzymes might be of importance.[964] However the direct intravenous injection of enzymes has not yet been used with success, probably because of the toxicity of the preparations employed. If the anti-trypsin of a tuberculous individual with a quiescent tubercle, encapsulated by scar tissue, should become reduced, the digestion of the protective connective tissue might conceivably lead to a liberation of the enclosed toxic material. On this hypothesis, measures to increase the anti-ferment content, such as selective diet, shock therapy, vaccines and the intravenous injection of lipoids, might be instituted but as yet there is no proof that the ferment-antiferment balance can be beneficially altered. It is possible that enzymes can be used in the surgery of old wounds, for the injection of pepsin solutions into scar tissue results in its softening; trypsin is of little value for this purpose.[957]

The oral administration of amylase (*Asp. oryzae* diastase), pepsin, and trypsin (pancreatin) preparations has often been recommended for digestive and other disturbances[90a] but there is no uniformity of belief in their efficacy, although it has been reported that the oral administration of pancreatin to infants suffering from diarrhoea reduces the number of stools and causes the patients to gain in weight.[918] Depancreatized dogs can be kept alive with insulin, but the animals suffer from diarrhoea unless raw pancreas is also fed. On the assumption that infants suffering from seborrheic eczema do not assimilate fat normally, these patients have been fed from 0.5 to 3 grams of pancreas tissue daily, in tablet or pulverized form, with apparently favorable results.[1061] Several proprietary infant foods are prepared by partial digestion with enzymes.

12. Use of Enzymes in Medical Diagnosis. The changes of the enzyme content of the blood, urine and faeces which occur during various diseases were discussed in Chapter V. The determination of these variations is sometimes useful as confirmatory evidence in the diagnosis of disease but the methods are still in the experimental stage. The measurement of the catalase content of the blood, excreta and tissues (see p. 79), the enzyme titer of the duodenum (see p. 67), the amount of diastase in the urine (see p. 85), and the differentiation of the type of lipase in the blood (see p. 76) are occasionally of some clinical assistance.

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